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RESUMO

O cancro é uma das causas mais comuns de morte nos países desenvolvidos. Infelizmente, a maioria das terapêuticas contra o cancro tem inúmeros e graves efeitos adversos, como citotoxicidade e danos nos tecidos saudáveis. Mesmo depois do tratamento, existem casos de resistência e recorrência. Contudo, a investigação nesta área está a tentar desenvolver terapêuticas que apresentem menos consequências e, o uso de nanopartículas (NPs) para a veiculação de fármacos, parece ser uma boa alternativa. Devido às suas propriedades físico-químicas excecionais, os nanotubos de carbono de paredes múltiplas (MWCNTs) estão a ser bastante utilizados na terapêutica anticancerígena. Têm a capacidade de veicular os fármacos diretamente aos tecidos tumorais por transporte passivo ou ativo, aumentando o potencial terapêutico, melhorando a segurança e biodisponibilidade dos fármacos administrados e diminuindo, significativamente, os efeitos adversos da quimioterapia.

Após a injeção de MWCNTs na corrente sanguínea, estes circulam até chegarem ao tecido-alvo. Uma vez em contacto com fluidos biológicos, proteínas e outras biomoléculas ligam-se à superfície dos MWCNTs, formando a proteína corona (PC). Os nanotubos de carbono conseguem alcançar as células tumorais pois os ligandos que estão na sua superfície reconhecem os seus recetores específicos que estão sobre-expressos nos tumores. Embora seja difícil simular as condições *in vivo*, nesta experiência *in vitro* juntámos nanotubos de carbono à albumina sérica bovina (BSA), originando complexos MWCNTs-BSA. Estes ganharam muita importância como transportadores para a veiculação direcionada de fármacos pois conseguem aumentar a estabilidade e o tempo de semi-vida dos antineoplásicos, prolongando o seu tempo na circulação, e regular a biodistribuição. É crucial para a maioria das aplicações clínicas das nanopartículas saber controlar a formação da PC. Para determinar a farmacocinética dos fármacos antes de serem aplicados na terapêutica antineoplásica, é necessário conhecer como a PC afeta a distribuição, metabolismo e eliminação dos MWCNTs. As interações entre os nanotubos de carbono e a albumina são essenciais para o comportamento da PC no corpo humano. Está provado que parâmetros experimentais como concentração de MWCNTs e albumina, tempo de incubação e temperatura podem influenciar a formação da proteína corona, sendo que o seu impacto precisa de continuar a ser investigado.

PALAVRAS-CHAVE: Nanopartículas anticancerígenas; Terapia alvo de medicamentos; Nanotubos de carbono com paredes múltiplas; Albumina; Proteína corona

ABSTRACT

Cancer is one of the most common cause of human death in developed countries. Unfortunately, most cancer therapies have numerous and serious side effects, such as cytotoxicity and damage to healthy tissues. Even after treatment, there are still cases of resistance and recurrence. However, cancer research is trying to develop therapies that have fewer consequences and the use of nanoparticles (NPs) for drug delivery seems to be a good alternative. Due to their exceptional physicochemical properties, multi-walled carbon nanotubes (MWCNTs) are widely used in anticancer therapy. They have the ability to deliver drugs directly to tumor tissues by passive or active targeting, increasing therapeutic potential, enhancing the safety and bioavailability of the administrated drugs and significantly decreasing the side effects of chemotherapy.

After injection of MWCNTs into bloodstream, they circulate until reaching the targeted tissue. Once into biological fluids, proteins and other biomolecules bind to the MWCNTs surface, forming the protein corona (PC). Carbon nanotubes can reach the tumors cells because the ligands on its surface recognize its specific receptor that are overexpressed in tumor tissues. Although it is difficult to simulate *in vivo* conditions, in this *in vitro* experiment carbon nanotubes were added to bovine serum albumin (BSA), originating MWCNTs-BSA complexes. These complexes have gained importance as carriers for targeted drug delivery because they can increase stability and half-time of anticancer drugs, prolonging the blood circulation time, and regulate biodistribution. It is crucial for most clinical uses of nanoparticles knowing how to control the PC formation. To discover what will be the pharmacokinetics of the drugs before it is applied in anticancer therapy, it is necessary to find out how PC affects the distribution, metabolism and elimination of MWCNTs. The interactions between carbon nanotubes and albumin to form PC are essential for their behaviour in a biological system, moreover, understanding the impact of the PC on biological response is crucial. Thus, it is proved that experimental parameters as concentration of MWCNTs and albumin, incubation time and temperature can influence the formation of protein corona and its impact needs to be further investigated.

KEYWORDS: Anticancer nanoparticles; Targeted drug delivery; Multi-walled carbon nanotubes; Albumin; Protein corona

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ACRONYMS

AA	Acrylamide
ABP	Albumin-binding protein
$AgNO_3$	Silver nitrate
ApoA1	Apolipoprotein A1
APS	Ammonium persulfate
Bis	Bisacrylamide
BSA	Bovine serum albumin
CH_3COOH	Acetic acid
CH_3OH	Methanol
CNT	Carbon nanotube
CTC	Circulating tumor cell
DNA	Deoxyribonucleic acid
DWCNT	Double-walled carbon nanotube
ECM	Extracellular matrix
EPR	Enhanced permeability and retention
Gp60	Glycoprotein 60
HSA	Human serum albumin
HCl	Hydrochloric acid
HCOH	Formaldehyde
HNO_3	Nitric acid
H_2SO_4	Sulfuric acid
IgG	Immunoglobulin G
MWCNT	Multi-walled carbon nanotube

Na_2CO_3	Sodium carbonate
$Na_2S_2O_3.5H_2O$	Sodium thiosulfate pentahydrate
NP	Nanoparticle
PBS	Phosphate buffered saline
PC	Protein corona
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPARC	Secreted protein, acidic and rich in cysteine
SWCNT	Single-walled carbon nanotube
TEMED	Tetramethylethylenediamine
Tris	Trizma base
TWCNT	Triple-walled carbon nanotube

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1 INTRODUCTION

1.1 CANCER: HISTORY, DEFINITION AND PREVENTION

Cancer history is long and extended back for many years before clinical signs evidence (1). It is difficult to trace the natural history of cancer but it is believed that cancer exists since the origin of humans (2). Cancer is one of the most common cause of death and the principal barrier to increasing life expectancy in the world today (3). In 2018, were diagnosed 18.1 million new cases and 9.6 million cancer deaths have occurred (4). It is expected 24 million new cases in 2035 (5).

Cancer is a group of diseases where a lot of cellular modifications occur. Currently, there are known more than 100 different types of cancer and not all cancers have the same impact, some are more deadly and aggressive than others (6). Carcinogenesis is a slow process where precancerous cells accumulate several mutations in the genes that participate in growth control, resistance to apoptosis and induction of angiogenesis in order to grow and invade multiple vital organs (7). These cancer cells can spread throughout human body by circulatory and lymphatic systems, causing metastases. In almost all cases, spontaneous tumors starts in a single cell (8). Cancers can be classified as *in situ*, local, regional, and distant based on the extent of spread (9). The conversion of a benign tissue into a malignant happens not only inside the cells but also in the tumor extracellular matrix (ECM) (10). Initially, cancer cells are not considered dangerous because they derived from normal cells, but their unregulated growth and dissemination to the host tissues give to these cells the capacity to induce significant morbidity and lastly mortality (11). Cancer incidence and the number of cancer survivors are rapidly growing world wide (3). The explanation for this phenomenon is complex but reflect both aging and growth of the population, because although cancer can appear at any age, the probability of developing cancer generally increases with age (11). It is also a result of earlier detection and treatment advances (9). The nature, start and course of cancer are controlled by genetics factors. Although cancers are recognized as spontaneous in nature, the truth is that the probability of an individual development a specific cancer can be inherited. The environment can increase the susceptibility of normal cells to oncogenic conversion, can mimic endogenous regulators of a growth promoting pathway, or inactivate a suppressor element working as a driver for cell growth (7). Another reason for the number of cancer cases increase is due to the prevalence of risk factors such as smoking, alcohol drinking, overweight and physical inactivity (12–16).

Cancer is probably the most preventable disease (17). To control the global problem of cancer, it is necessary to combine efforts and focus on primary and secondary prevention as also in treatment and palliation (5). In fact, one-third of tumors could be preventable if vaccines were universalized (18,19). At the first sign of infection or other inflammatory situation that leads to changes on the normal tissue homeostasis, immune system can recognize antigenic molecules produced by abnormal cells. However, cancer cells can escape to immune system once they can acquire antigenic and other changes. To confront this problem, the administration of vaccines based on tumor antigens at pre-malignant stages of cancer could be the solution, because they will act before tumor-associated immune suppression is settled. These preventive vaccines should have pre-malignant lesions as target, eliminating them, preventing their recurrence after surgery or even preventing their progression to cancer. It was proved that if a vaccine to prevent cancer was given in infancy, it would give protection in adulthood (20). For cancer immunotherapy, the development of cancer vaccines should be from whole tumor cells and when used for postoperative cancer therapy would have a great potential (21). Also alcohol and tobacco control programs are needed as part of preventive measures, once they can help to eradicate alcohol and tobacco related cancers, such as lung and oropharinge cancer (5,14). Research on cancer prevention is very important not only because it is necessary to decrease human suffering due to cancer diagnosis and treatment but also because it is an economic requirement (20). Newly diagnosed cancer patients could experience increased survival or early-stage detection. Metastatic biopsies are often used to diagnosis and to characterize the primary cells. The problem here is that metastatic and primary cancers can be different and these differences are important in terms of therapy (7). Precancerous cells have little genetic differences and the absence of an appropriate blood supply turns the cells much more vulnerable to anticancer molecules than mature tumor cells would become. Once carcinogenesis takes several years, gives a large therapeutic window to stop cancer progress (6). When cancer is diagnosed early, patients have less complications and more chances to survive than those whose cancer is diagnosed in a late phase (22). Some staging systems are used to classify cancers. The TNM staging system is based on the size and extension of the tumor (T), regional lymph node involvement (N), and the presence of distant metastases (M), and sometimes with more details (9). Diagnosis of most cancers is frequently made in advanced stages (III and IV), and treatment options are limited and expensive, so early diagnosis is mandatory to improve prognosis (23). The most important tool available to have long-term successful outcomes is early diagnosis, but areas such as physical activity and obesity progress are also very important (5,7).

1.2 CANCER TREATMENT: OPTIONS AND DIFFICULTIES

Although cancer incidence is growing, it is evident that cancer treatment is improving. The improvements that are made are possible due to different efforts made in prevention, early detection, and improved therapeutic strategies, but sometimes treatment efforts are not enough once exists many causes and characteristics of cancers (7). Cancer cure is difficult to achieve, however preventing metastasis through early detection and ceasing cancer cells with cancer drugs are the most efficacious therapies. It is necessary various levels of investigation to make advances in cancer treatment and anticancer drugs need to pass over *in vitro* testing, *in vivo* animal experiments and finally clinical studies (2). When a new anticancer drug is produced by a pharmaceutical company, their biggest demand is testing its efficacy and safety (24).

Nowadays exists different therapeutic approaches and cancer can be treated by surgery or with radiotherapy, chemotherapy, adjuvant chemotherapy, neoadjuvant chemotherapy or immunotherapy (11).

1.2.1 THERAPEUTIC APPROACHES

1.2.1.1 SURGERY

The first option for clinical treatment in cases of nonmetastatic solid tumors and early stage is surgical resection (21). For tumors that are in places which are surgically accessible, surgery is the elected treatment (25). Nevertheless, lethal tumor recurrence and metastasis after months or years postoperation can occur and be caused by residual microtumors or circulating tumor cells (CTCs). The recurrence of the tumor at the primary local is the cause for primary surgical treatment failure (26).

1.2.1.2 RADIOTHERAPY

Ionizing radiation is highly effective at inducing biological effects but it can be a potential health threat, however can be profitable when used to radiotherapy (27). Radiation biological effectiveness depends critically on the concentration of oxygen (28). In situations where oxygen is present, cells and tissues are significantly more sensitive than when they are at hypoxic conditions (27). The deoxyribonucleic acid (DNA) is the target for a range of biological endpoints of the radiation. Cells with deficiencies at the level of the DNA are more sensitive to damage by the ionising radiation (27,29). Nowadays, radiotherapy is used more selectively (25). When radiotherapy is used it is required extreme caution to find the balance between the necessary dose to cure the patient and the maximum dose that will be tolerated to

avoid toxicity at the surrounding tissues (27). For most patients, to be an effective treatment it is necessary to achieve every organ at their body (30).

1.2.1.3 CHEMOTHERAPY

The chemist Paul Ehrlich had the idea of developing drugs in order to treat infectious diseases, creating the term "chemotherapy" as the use of chemicals to cure cancer cells (31,32). The modern era of chemotherapy began when it was discovered nitrogen mustard as an option for cancer treatment (33). It was demonstrated that this reagent forms a covalent bond with DNA at specific sites of alkylation on purine bases, conducting to crosslinking of strands and induction of apoptosis (30). This therapy is used to prevent proliferation of cancer cells, prevention of metastases formation and, later, to kill all the affected cells (34).

It is assumed that tumor cells are more sensitive to those drugs than normal cells (30). Although this therapy is one of the most used at cancer treatment, it can not be totally successful, due to drug resistances (35). In present days, chemotherapy is more used in cases of earlier stages of tumors and for metastatic and locally advanced cancers complemented by its surgical resection (36).

1.2.1.4 ADJUVANT CHEMOTHERAPY

Surgery and radiotherapy were the most common cancer therapies used until studies have revealed that the combination with chemotherapy could treat and cure more patients with different advanced tumors. The concept of adjuvant chemotherapy appeared when it was demonstrated that the combination of drugs with surgery and/or radiation treatments could cure patients with micrometastases. At the same time, when chemotherapy is used as an adjunct to surgery or radiotherapy it is mandatory to prove that the drug is not excessively toxic and will not expose the patient to potential side effects of chemicals, unnecessarily (31). Adjuvant chemotherapy is usually used for patients with high risk of recurrence after surgical resection (37).

1.2.1.5 NEOADJUVANT CHEMOTHERAPY

In order to achieve a better result with surgery, maintaining the vital organ functions and reduce the size of the tumor, chemotherapy is being used as a neoadjuvant (31). For post-surgical cancer management, neoadjuvant chemotherapy and radiotherapy are currently used (21).

1.2.1.6 IMMUNOTHERAPY

Cancer chemotherapy can kill cells that are dividing more rapidly but have significant toxicities and commonly develop resistances. Recently, with the knowledge acquired about cancer pathogenesis, new treatments are being explored and tested, such as cancer immunotherapy (21). Immunotherapy for cancer treatment was proved that have results not only in cases of recurrent or regressive tumors but also with metastatic tumors, by increasing host anti-tumor immunities (21,38). Even though pathogens are more immunogenic than cancer cells, immune system can easily recognize and eliminate tumor cells. Yet, tumor cells often interfere with the role of immune responses, so immunotherapy is continuously studied and new strategies are being created to guarantee effective and safe responses. Some regimens for cancer treatment are already being done by the administration of monoclonal antibodies and cytokines (39). With the formation of antibody-drug conjugates it is possible to achieve a highly selective and cytotoxic cancer treatment with a large therapeutic window (40,41).

1.2.2 GOALS OF THERAPY

Patients can do cancer therapy for many reasons. With the treatment, it is possible to cure the cancer, destroying all the tumor cells, or just prolong survival in cases of advanced disease. Cancer treatment is also used as metastasis prophylaxis or as palliative care, trying to preserve quality of life (42,43). When the treatment is done with chemicals, they can damage cells of sensory nerve, leading to peripheral neuropathy. This damages are dependent on the dose applied and can take months to years to treat (43). Most patients that are doing active treatment also need pain control, to give them enough relief to endure the diagnostic and therapeutic treatments (44).

1.2.3 ANTICANCER DRUGS: PROBLEMS AND SOLUTIONS

A relevant part of cancer care is the management of symptoms, side effects and toxicities from cancer treatment. It is important because it will minimize suffering and maximize quality of life during the progress of cancer (45). Chemotherapy is supposed to kill the maximum number of tumor cells and, at the same time, it is expected a certain degree of toxicity at normal tissues, because anticancer drugs act not only on cancer cells but also on normal cells (34,37). In fact, the majority of cytotoxic chemotherapy drugs cause side effects and most patients submitted to those therapies experienced some side effects because these drugs have poor selectivity for cancerous cells over normal cells (45,46). The tumor environment is acidic and can ionize the drugs, preventing their diffusion across cellular membrane, leading to a resistance

mechanism against basic drugs. The extravasation of molecules can be retarded or blocked due to the difference between the low microvascular pressure and high interstitial pressure. Cancer cells and tissues have high nutrient requirements which will lead to this low selectivity. Although this chemicals are absorb by cancer cells that are growing rapidly, other tissues that also grow fast are sensitive to them (37,47). Mucous membranes of the mouth, stomach and intestines are tissues that grow fast, so it is normal gastrointestinal toxicity which will cause nausea and vomiting. Hair follicles are one of the most sensitive tissues to chemotherapy drugs, so is very typical hair loss, and this includes eyebrows, body and facial hair and ear hair, which can provoke ototoxicity. Bone marrow is also affected which leads to a low production of red and white blood cells and myelosuppression is one of the most common side effects. Chemotherapy can also provoke damage at liver and kidney during the metabolization, detoxification and excretion of the drugs (37,46). These side effects can be acute and short lived or can continue and turn in to chronic. In some cases, they can appear months or even years after treatment ends and, in this situation, they are described as late effects. The clinical factors, such as cancer type and therapy, and the patient characteristics, like sex and age, will determinate the type and prevalence of the side effects (9). Pain, nausea and vomiting, hair loss, emotional distress and fatigue are the most commonly symptoms experienced by patients (48,49). Anyways, the ability of the treatment to target and to kill cells with cancer while affecting as few healthy tissues as possible is directly related to the effectiveness of the treatment (47).

It is required that the side effects of chemotherapy are manageable. Regional chemotherapy can be a solution, because with the delivery of cytotoxic drug directly to the tumor tissue, the toxic effects to the healthy drugs are reduced and the efficiency of the therapeutic is maximizing (2,34,50). Drug-delivery systems can be helpful with drugs that do not have good physicochemical properties. To overtake the high hydrophobicity of most of anticancer drug, co-solvents, polymer micelles, liposomes and hydrotropic agents are joined to increase their water solubility. Delivery systems in nanosize, also known as nanovehicles, are a great way to deliver anticancer drugs more specifically, although the amount that is delivered is still small. Nanovehicules can release the drug at the right tissues because they are responsive to environmental changes in pH and temperature. Testing these systems *in vivo* is very complicated, so there is a high need to develop an *in vitro* model systems that will replicate *in vivo* conditions faithfully. These *in vitro* models are very important to understand the mechanisms of, not only drug delivery systems, but also drug action (2).

1.3 ANTICANCER NANOPARTICLES

Nanotechnology is a multidisciplinary field that utilizes nanomaterials for various biomedical applications. The interest in this area is increasing due to their capacity to change the pharmacokinetics of the drugs (51–53). Nanomedicine is a crescent medical field that uses nanoparticles (NPs) for new applications in medicine, including the diagnosis and treatment of cancer by carrying many diagnostics and therapeutic agents (54,55). Nanoparticles include particles that have dimension between 1 and 100 nanometres (nm) and due to their small size, they have access and can enter in almost every part of the human body, including organs, tissues and organelles, by different ways of administration, such as injection and ingestion (54,56–58). The control of surface properties of nanoparticles is crucial to expect their behaviour in human body due to their high surface-to-volume ratios. Nanoparticles in the size range of 10-100 nm and with their surface charge either slightly negative or slightly positive, when administrated into the circulatory system, have access to and within disseminated tumors (59). Nanoparticles are a promising way for the targeted delivery of anticancer drugs to the desired tissues and cells in the human body (60).

The pharmacokinetics of anticancer drugs are changed due to nanomedicines, because they can extend plasma half-life and provide different biodistribution profile. Nanoparticles can dissolve poor soluble drugs in their hydrophobic and hydrophilic compartment (61). Thus, they can increase the solubility and stability of the drugs, prolong circulation in the bloodstream, reduce their metabolism, control the release of the drug and prevent the degradation of acid-sensitive drugs that occurs due to the acidic tumor microenvironment (53,62).

Overtaking the barriers, it is supposed that nanoparticles reach the tumor tissue with minimal loss of activity and volume, enter inside cancer cells and start to produce cytotoxicity (53,63,64). To achieve the tumor tissue by systemic circulation, nanoparticles have two different strategies: passive and active targeting, that are represented in Figure 1 (53).

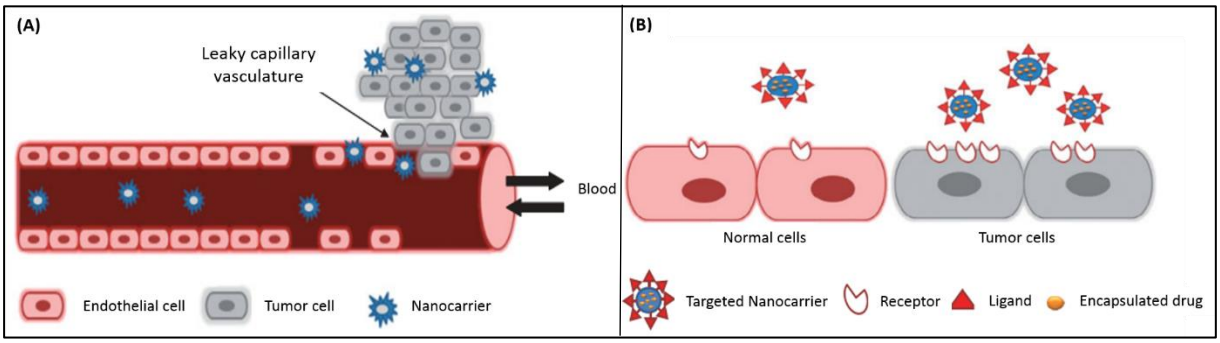

Figure 1. Schematic representation of passive (A) and active (B) targeting utilized by anticancer nanoparticles (53).

1.3.1 PASSIVE TARGETING: ENHANCED PERMEABILITY AND RETENTION EFFECT

In cancer therapy, nanoparticles are very advantageous because they have the ability to be accumulated at tumor tissues due to the enhanced permeability and retention (EPR) effect of tumors. Anticancer nanoparticles entry and stay in tumor tissues by this effect with much stronger activity than free drugs (65,66). The ineffective lymphatic drainage and defective vasculature of tumor interstitium leads to a passive accumulation there by the EPR effect (67). When compared with normal and healthy tissues, most of solid tumors have a higher vascular density (hypervascularity) and the blood vessels that are around tumor tissues present larger pore sizes, which leads to a preferential tumor accumulation of anticancer nanoparticles, higher treatment efficacy and lower systemic toxicity (65,67,68). Nanoparticles accumulated in tumors depend on several factors, such as the size, surface characteristics, shape and the degree of angiogenesis of the tumor, once angiogenesis is a crucial factor for progression of tumor (65). Their physicochemical properties can allow them to avoid renal clearance, reach to tumor cells in sufficient amounts, undergo active cellular uptake and, finally, induce a biological response with almost none non-specific interactions (61).

Basically, by passive targeting, anticancer nanoparticles enter in the tumor interstitial space thanks to the high permeability of the tumor vasculature and due to the compromised lymphatic filtration, they can stay there. In contrast, in the normal endothelial cells the tight junctions do not allow such extravasation (66,69). In fact, EPR effect is considered a promising way for anticancer drug delivery, however, faces several challenges in delivering drugs to the tumor sites (53,70,71).

1.3.2 ACTIVE TARGETING

Compared to passive targeting, the other strategy, active targeting, presents more advantages for directing the nanoparticles to tumor site (53,64,72). The active targeting of NPs occurs when the nanoparticles are targeted to a desired location (61). This strategy is possible by several ways. One of the most common targeting strategies is when the nanoparticles have specific ligands in their surface, such as proteins, for recognition of specific receptors in tumor. It is known that in a wide range of cancer cells, their surface has more receptors of proteins. Another strategy is based on stimuli-sensitive drug targeting, in which nanoparticles will respond to small environmental changes and provoke drug release (73,74). The presence of these targeting ligands in nanoparticles enables them to bind to cell-surface receptors. With this binding, NPs enter in the cells by receptor-mediated endocytosis and this leads to an

accumulation in tumor tissues (59,61). This system of active targeting have six times higher drug delivery efficiency when compared with drug in its free form, due to their capacity of loading sufficient drug and prolonged release (34).

1.4 PROTEIN CORONA: INTERACTIONS BETWEEN ALBUMIN AND CARBON NANOTUBES

1.4.1 CARBON NANOTUBES

Recently, carbon nanotubes (CNTs) have been widely used in many electronics, medicine, space and military applications due to their outstanding properties (73). Carbon nanotubes are well-ordered and can be classified, depending on the number of graphene layers, as single-walled carbon nanotubes (SWCNTs), double-walled carbon nanotubes (DWCNTs), triple-walled carbon nanotubes (TWCNTs) or multi-walled carbon nanotubes (MWCNTs) (75).

These one-dimensional carbon allotropes consist of high surface area, high mechanical strength, ultra-light weight, rich electronic properties, and excellent chemical and thermal stabilities (76). Because of the cell-penetrating ability, sustained-release and drug targeting, CNTs are expected to be the basis for potential biomedical and biotechnological applications, in particular, used for drug delivery once they can enter in all sorts of cells (65,77). It is possible to achieve the cancer tissues effectively, internalize CNTs into target cells and deliver the drug into the cytoplasm due to their nano-needle penetration technique (34,65).

MWCNTs are considered as new carriers for drug delivery and diagnostic applications. Their unique physicochemical allows the non-covalent and covalent introduction of different drugs and they are a novel candidate for the delivery of a wide range of therapeutic agents (75). Once MWCNTs are a recent type of nanomaterial, their potential toxicity has been intensively investigated not only *in vitro* but also *in vivo* (53).

1.4.1.1 TOXICITY OF CARBON NANOTUBES

Over the past decade, biological implications of CNTs exposure have received more attention. At the beginning, many studies done in *in vitro* conditions revealed that CNTs could enter inside mammalian cells by penetrating their plasma membrane and could cause cytotoxicity. However, the toxicity of CNTs is attenuated after the binding of serum proteins because, with the formation of protein corona (PC), the proteins that are in the surface of CNTs have saturated their protein binding sites and thus prevent more interactions (76). Until CNTs

and PC are eliminated through phagocytosis, the presence of protein corona avoids the damaging effects that the bare nanoparticle surface could cause in the cells (78).

1.4.2 FORMATION OF PROTEIN CORONA

There is a gap between the *in vivo* and *in vitro* studies due, in part, to the adsorption of proteins in the surface of nanoparticles when they are expose to biological fluids, mostly blood (60,79). There are more than a thousand molecules in the blood like proteins, lipids and nucleic acids. Once NPs are introduced into organism and arrive to the bloodstream or tissue interstitial fluids, proteins, small molecules and ions will compete to absorb on the surface of nanoparticles, through non-covalent interactions, due to its high reactivity (80). Also, once injected in human body, nanoparticles cross several compartments that exhibit different protein concentrations and compositions, temperatures, pH values and ionic concentrations (81). Although it is desired that NPs have a long blood circulation time in the body and this is a prerequisite for targeted drug delivery, the accumulation of the NPs loaded with the drug at the tumor tissue is more relevant (79). PC can be used not only to load drugs but also can reduce significantly the burst effect of nanoparticles (78).

CNTs, like other nanoparticles, will absorb different proteins and form the PC (76). Immunoglobulin G (IgG), fibrinogen, human serum albumin (HSA) and apolipoprotein A1 (ApoA1) are the four most common serum proteins (82). The proteins that constitute the PC are affected by several factors: NP shape, NP size and various surface properties of CNTs, such as surface functional groups, surface hydrophobicity/hydrophilicity and topography of the surface. Also, PC can be affected by some experimental parameters as pH, incubation time and temperature (83). The impact and influence of these factors on the formation of PC still need to be investigated. When PC is formed, proteins can modify their structures to adapt to CNTs surface and surrounding environment. These alterations are known as “conformational changes” and the secondary and tertiary structures of the proteins can be modified. Also, this can lead to changes on the physicochemical properties of CNTs such as size, surface composition, surface charge and functionality (56). Protein corona has been shown to influence nanoparticle cellular uptake, trafficking, opsonization and biodistribution, leading to changes in the circulation time, enhance the stability and control the biodistribution of CNTs. Beyond these biological changes, PC can compromise the targeting efficiency of CNTs (60). The distribution of this complex depends on the nanoparticle charge, and an example of that is the rapid degradation of PC towards gastric or intestinal fluids (80). If PC has in its surface

complement factors, IgG or fibrinogen, it will be rapidly removed from the body. In contrast, if protein corona is covered with albumin, the circulation time is longer (54,84). It is proved that the interactions between proteins and CNTs have several consequences and can provoke a giant impact on their pharmacological activities and lead to different biological responses. The formation of PC largely defines biological identity of nanoparticles and it is believed that this complex is more significant to determine the biological response than the properties of nanomaterials without the coating (57,79). Proteins can bind to CNTs with low or high affinity, creating a soft or hard corona, respectively. Soft corona is created by lower affinity proteins and this bound is reversible, which makes them easier to separate by washing steps during experiments. In contrast, hard corona has higher affinity proteins on the surface of CNTs and this bind may be irreversible (57,78,79). PC will determine the physicochemical behaviour of the CNTs and its properties are more important to determine the biological response, than the properties of CNTs. It is crucial for most clinical uses of nanoparticles knowing how to control the formation of PC. To find out what will be the pharmacokinetics of the drugs before it is applied in clinical practice, it is necessary to find out how PC affects the distribution, metabolism and elimination of CNTs (80).

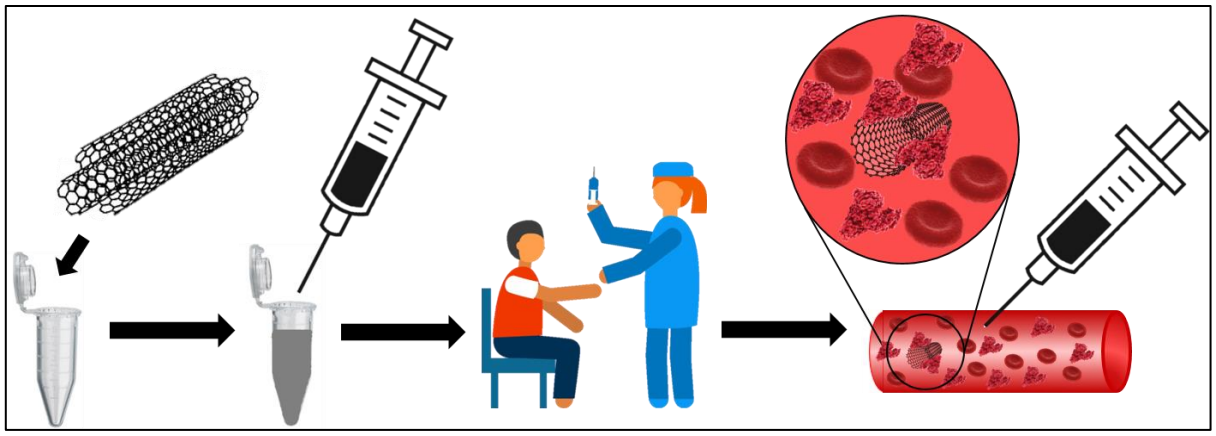

Figure 2. Suggested scheme of protein corona formation. MWCNTs are placed in the reaction solution. The antitumor drug binds to the surface of the nanotransporter and is injected into the pacient. When contacts with blood, forms the protein corona.

1.4.3 HUMAN SERUM ALBUMIN

The most abundant protein in blood is human serum albumin (HSA) (79). This protein is predominately present in the extravascular space, such as skin, muscle, secretions and gut, instead of the intravascular space. Just a small concentration of albumin is present intracellularly. This protein is very important to maintain osmotic pressure and is also crucial in different processes such as transportation of drugs and detoxification. Albumin is usually one of the residents in the corona formed around CNTs and act as surface active agents/ surfactants

by binding to nanoparticles. Albumin is a non-glycosylated and negatively charged protein and has been used as a drug carrier. Using CNTs covered with albumin has several advantages because albumin is an endogenous protein and due to this, they can enhance the bioavailability and biodistribution of the anticancer drug. Besides the fact that they are non-toxic and non-immunogenic, albumins are highly soluble, promptly available, have a long circulation time and their surface can easily be manipulated and modified (80).

Bovine serum albumin (BSA) is the most abundant protein in bovine blood and presents a very similar structure with HSA. BSA is commercially available and has numerous uses in several areas such as nanocarrier development and drug delivery, due to its exceptional biocompatibility. As a transport protein, BSA can binds reversibly with numerous endogenous and exogenous drugs (85). BSA is an appropriate model system for the study of interactions between nanoparticles and proteins and is often used in experiences in place of HSA (57).

1.4.4 DRUG DELIVERY

After secretion from hepatocytes, this protein enters in circulation and goes to the extracellular space through the fenestrated endothelium in some organs, like pancreas, liver, bone marrow and small intestine. So, due to EPR effect, albumin-based NPs can be readily accumulated in tumor tissues. It is believed that in organs without slots, which have a continuous endothelium, albumin can pass through it by active transcytotic mechanisms, like receptor-mediated mechanisms. Because of the high necessity of nutrients and to satisfy the energy and materials that cancer cells need, their nutrient transporters are overexpressed. There are a few membrane-associated albumin-binding proteins (ABPs), such as albondin, or glycoprotein 60 (gp60), and secreted protein, acidic and rich in cysteine (SPARC) that are overexpressed in malignant, stromal and tumor vessels endothelial cells. Thereby, the uptake of albumin is increased in tumors due to their interaction with albondin and SPARC. This tumor uptake is dependent of the tumor size, so bigger tumors have higher albumin uptake. Albondin, a 60 kDa glycoprotein that acts as a receptor of albumin and is selectively expressed on the plasma membrane of continuous endothelium, helps the internalization of albumin, facilitating their accumulation in tumors, and subsequent transcytosis. SPARC is secreted by different cells and is highly expressed in malignant cells and stromal cells with neoplasia. These two proteins exhibit a good albumin-binding properties and interacts with albumin similarly (54,79,80,84,86,87).

Albumin is an excellent carrier protein that has the ability to surpass barriers through different uptake pathways to deliver small molecules, such as anticancer drugs (88). Albumin is the principal source of energy and nutrition for the growth of tumors (86,87).

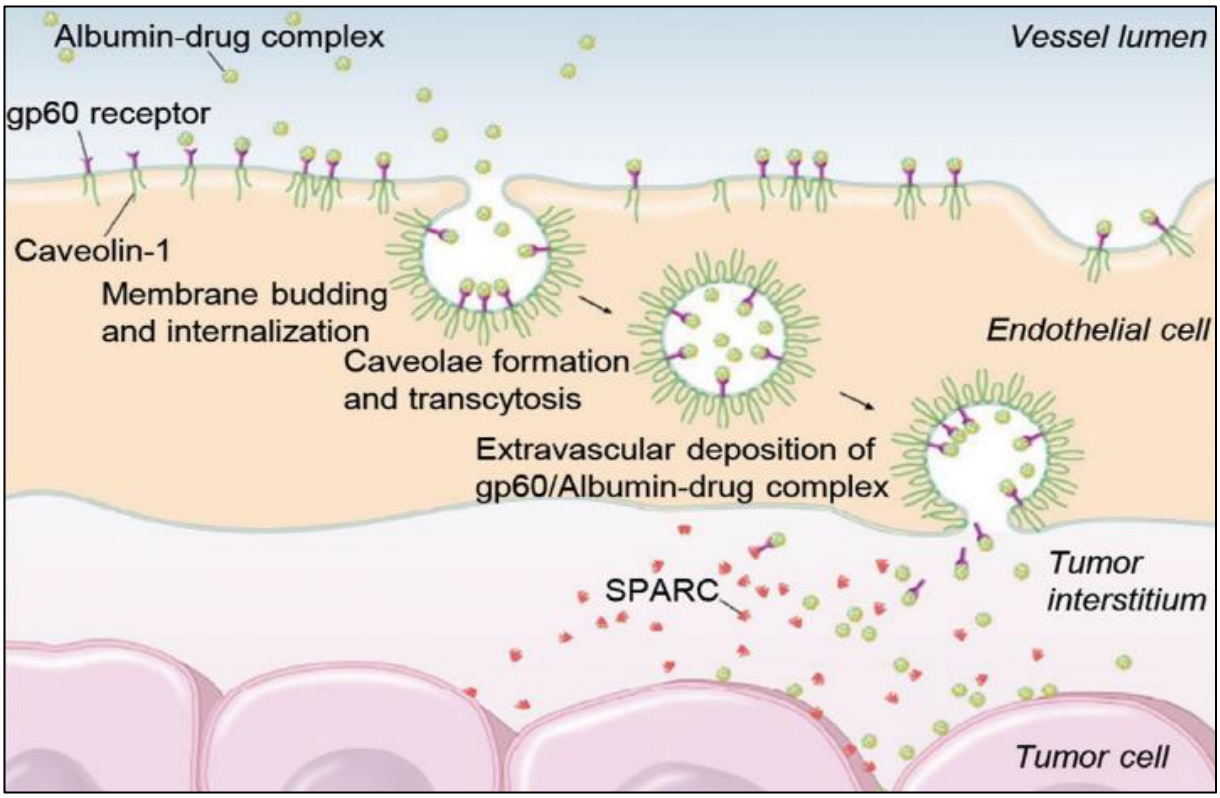

Figure 3. The uptake of albumin via albondin (gp60) by the transcytosis pathway. After this, albumin binds to SPARC that are overexpressed in tumor cells (87).

1.4.5 DRUG RELEASE

The formation of PC is a relevant consideration to achieve an effective drug release. The drug release profile is defined by the properties of CNTs and the experimental conditions to the formation of PC (78). The release of drugs that are inside the PC can be started naturally by protease digestion or in response to pH changes. To evaluate the interactions between CNTs and proteins, pH condition are relevant factor to be considered because alterations of environmental pH can lead to changes in protein binding affinity and consequently can modify the adsorbed protein pattern. Once in the bloodstream, CNTs are biodistributed to different organs with different pH. Tumor tissues and cancer cells not only present an acid microenvironment but also have certain types of proteins which can alter the PC around CNTs, leading to changes in bioavailability and therapeutic effects and responses (79). Furthermore, depending on the amount of albumin on the surface of CNTs, it is possible to alter the quantity of drug that are released (80).

2 OBJECTIVES

After given the current scenario of cancer worldwide and noticing the high incidence of these diseases nowadays, it is possible to realize the importance of early diagnosis and the necessity of better treatment approaches due to their side effects. Using nanoparticles as nanocarriers of anticancer drugs present a hope for improving cancer therapy once they act at two main levels: give new and better properties to the drug and drive the pharmaceutical agent directly to the tumor cells. When NPs are introduced in human body, proteins such as albumin will cover their surface and PC is formed. Considering the EPR effect and the high accumulation of albumin in tumor cells, the development of albumin as a drug carrier is essential in terms of the targeted delivery of anticancer drugs. Furthermore, PC is effective to prolong the CNTs circulation time and to reduce their cytotoxicity (67).

Therefore, the major aims of this work are to understand the interactions between CNTs and albumin, forming the PC, and the influence of environment conditions.

Considering the nature and objective of this experiment, it was simulated the *in vivo* conditions at the laboratory. For this purpose, oxidized MWCNTs were added to BSA, to replicate the administration of CNTs in the bloodstream. To understand the influence of CNTs and protein concentrations, pH and temperature in the formation of PC multiple samples were prepared and exposed to different environment conditions.

3 MATERIALS AND METHODS

3.1 MATERIALS

Basic laboratory plastic consumables (tips and microtubes) were purchased from Eppendorf (Germany). Pipetting was carried out by pipettes Eppendorf (Germany). Multi-walled carbon nanotubes (MWCNTs), sulfuric acid (H_2SO_4), nitric acid (HNO_3), bovine serum albumin (BSA), phosphate buffered saline (PBS), trizma base (Tris), glycine, sodium dodecyl sulfate (SDS), hydrochloric acid (HCl), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), acrylamide (AA), bisacrylamide (Bis), bromophenol blue, glycerol, acetic acid (CH_3COOH), methanol 95% (CH_3OH), formaldehyde 37% (HCOH), sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$), silver nitrate ($AgNO_3$) and sodium carbonate (Na_2CO_3) were purchased from Merck (USA). Pyrogallol red and Biuret reagent were acquired from SKYLab Svitavy. Distilled water was prepared by the Aqual system (Czech Republic) and ultra-purified water (Milli-Q water) was prepared by the ELGA (USA) system to a sterile 18 MΩ quality. Weighing was done on BOECO Analytical Weight (Germany). Control and pH adjustment of the solutions was performed on the Inolab multimeter (Germany).

3.2 METHODS

3.2.1 PREPARATION OF MWCNTS

Multi-walled carbon nanotubes have a unique structure, exceptional mechanical strength and essential physicochemical properties, enhancing their viability for many uses (89). Commercially supplied MWCNTs do not exhibit functional groups on their surface, therefore it becomes necessary to adjust their surface and shorten their length for their further use in biomedicine. The MWCNTs that were used in the experiment had a diameter between 10–20 nm and a length between 5–15 μm. Firstly, 5 mg of MWCNTs suffers a chemical oxidation at high temperature (75 ºC) in the heating mantle for 7.5 hours under reflux and stirring at 300 rpm in the environment of nitric acid and sulfuric acid in a ratio of 1: 3. The MWCNTs oxidized were subsequently treated by ultrasound (60 min, 40 W). The effect of ultrasound consists in the distribution of individual nanotubes, thus improving surface accessibility to further interventions. MWCNTs were increasingly dispersed in the mixture and the course of the reaction could be monitored visually. Carboxyl functional groups were formed on the surface of the nanotubes. After concluding the carboxylation, reaction solution and MWCNTs were washed with 18 MΩ ultra-pure water in centrifuge (30 min, 14000 rpm). This step was repeated 3 times. Subsequently very well dispersed MWCNTs in water were obtained. Oxidized

MWCNTs (1 mg/mL) were monitored for 10 days in 2 mL tubes. The experimental arrangement is summarized in Figure 4.

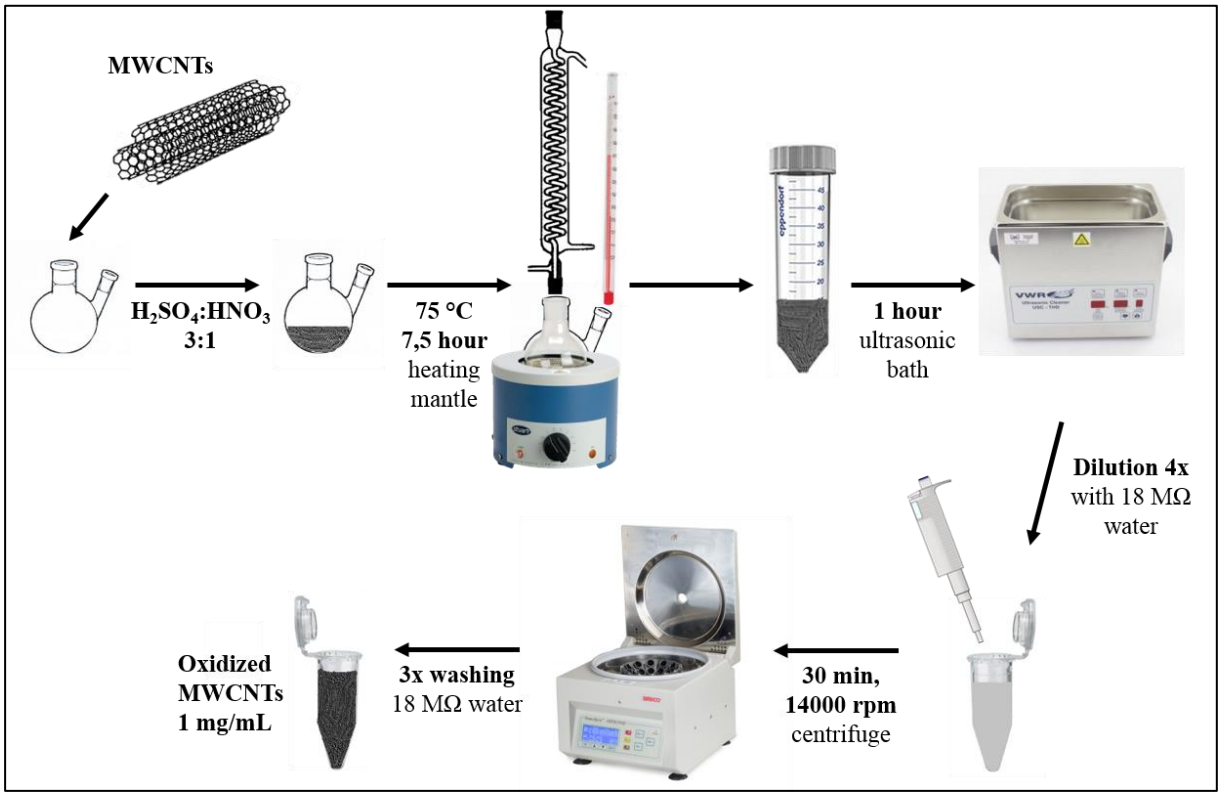

Figure 4. Experimental scheme of multi-walled carbon nanotubes preparation.

3.2.2 FORMATION OF MWCNTS-BSA COMPLEXES

Firstly, stocking solutions of 100 mg/mL BSA in PBS and 1 mg/mL MWCNTs in PBS were prepared. It is known that environment conditions are important to the formation of MWCNTs-BSA complexes. To explore the influence of concentrations in the formation of these complexes, different concentrations of BSA and MWCNTs were bound in Eppendorf tubes. These tubes were exposed to 3 different temperatures for scheduled times.

Samples with different BSA concentrations were obtained from 100 mg/mL BSA solution, as follows: 0 mg/mL (0 µL), 15 mg/mL (300µL), 30 mg/mL (600µL), 45 mg/mL (900µL) and 60 mg/mL (1200µL). To each one of these 5 Eppendorf tubes, MWCNTs were added and the volume completed until 2 mL with PBS. After this, from these 5 tubes, 0.5 mL were transferred to new tubes and submitted to centrifugation for 15 minutes at 14000 rpm. After this time, the samples were washed with PBS and centrifugated again for 15 minutes more. This method was repeated for 3 times to guarantee that all the BSA not bound to MWCNTs was eliminated. The remaining 1.5 mL were incubated on the thermoblock with agitation. After 15 minutes, another 0.5 mL were removed from the tubes that were on the

thermoblock and submitted to centrifugation. This procedure was repeated after 30 and 60 minutes. MWCNTs-BSA complexes had different incubation times. Samples were exposed to 3 temperatures: 30 ºC, 37 ºC and 45 ºC. Samples with different MWCNTs concentrations were obtained from 1 mg/mL MWCNTs solution and 3 different concentrations were prepared: 200 µg/mL (400µL), 100 µg/mL (200µL) and 50 µg/mL (100µL). The followed table (Table 1) demonstrate the 20 samples prepared to each concentration of MWCNTs.

Table 1. Composition of prepared samples with 200, 100 and 50 µg/mL of MWCNTs, different volumes of BSA and with 4 distinct incubation times at 30 ºC, 37 ºC and 45 ºC in the thermoblock.

CONCENTRATION OF CNTS	**CONCENTRATION OF BSA**	**INCUBATION TIME**	**TEMPERATURE**
µg/mL	**mg/mL**	**minutes**	**ºC**
200 or 100 or 50	0	5’	30
	15		
	30		
	45		
	60		
	0	15’	
	15		
	30		
	45		
	60		
	0	30’	
	15		
	30		
	45		
	60		
	0	60’	
	15		
	30		
	45		
	60		

The same technique preformed with the previous 60 samples illustrated in Figure 5 was repeated exposing them to 37 ºC and 45 ºC on the thermoblock. In total, 180 samples were prepared.

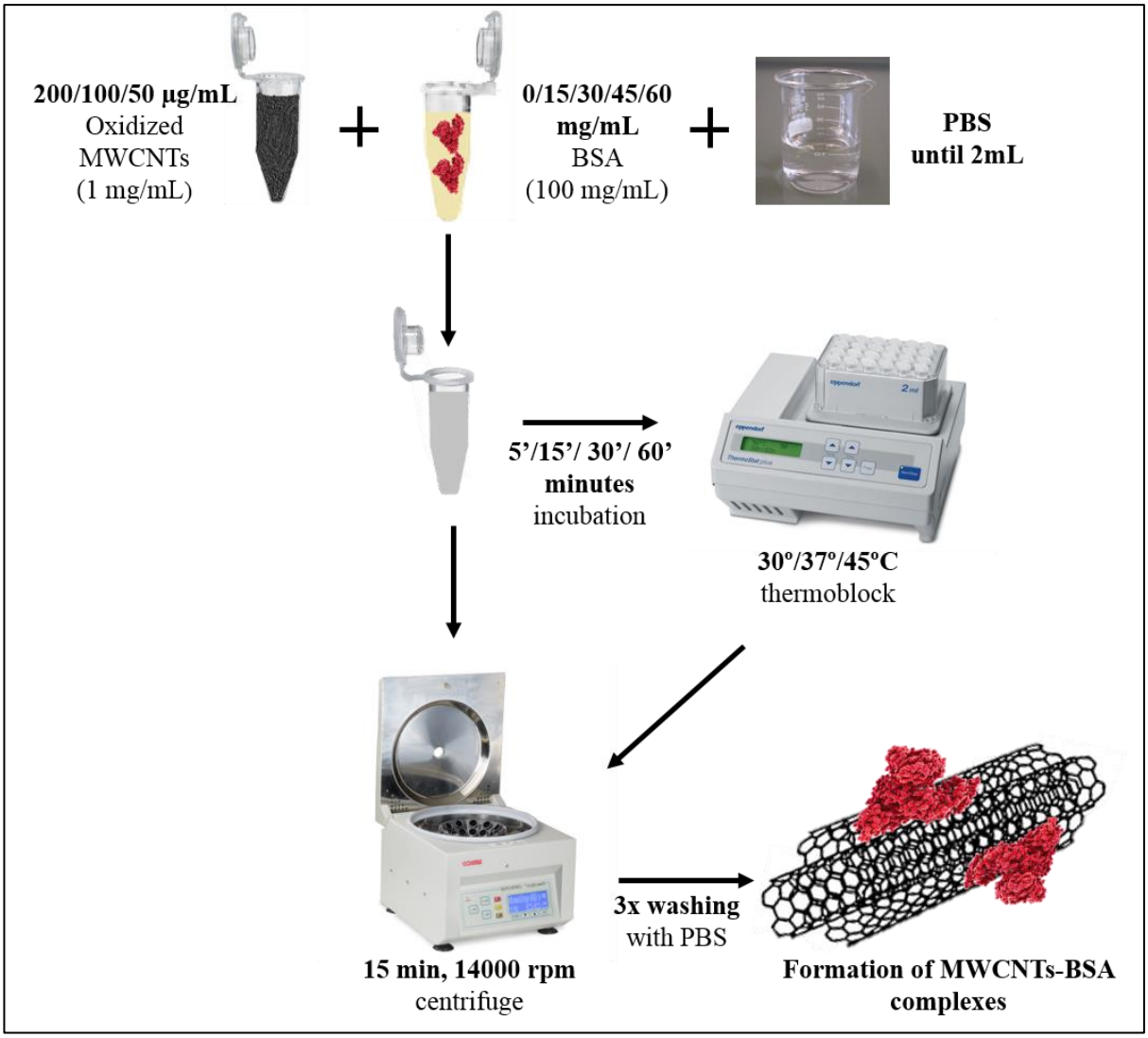

Figure 5. Schematic illustration of MWCNTs-BSA complexes formation in *in vitro* conditions.

3.2.3 PROTEIN CORONA ANALYSIS

3.2.3.1 ELETROPHORESIS

Gel electrophoresis is a common technique used for the fractionation of complex protein mixtures by electrical charge. The separation of proteins is based on their charge and molecular weight (90). Electrophoresis is a very accurate separation method and the driving force is the movement through an electric field. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is a typical workflow used in the analysis of enormous range of samples (84,91,92). Due to the specific migration time is possible to identify the proteins by

this method (93). After the formation of MWCNTs-BSA complexes, the presence of PC was analysed by 15% SDS-PAGE gel vertical electrophoresis system (Shirogel, VWR, Germany) in 1x running buffer (Table 2) with a voltage set at constant 150 V ((E0322 power supplier, VWR, Germany) for 60 minutes. In brief, equal volumes (10 µL) of each 180 samples were mixed with 5 µL of loading buffer (Table 3) and loading in to the wells of an 15% SDS-PAGE gel that was formulated according to the instructions describe in Table 4.

Table 2. Recipe for preparation of running buffer in a 1000 mL volumetric flask.

RUNNING BUFFER	
Trizma base	3.02 g
Glycine	14.4 g
SDS	1 g
Milli Q water	Until 1000 mL

Table 3. Recipe for preparation of loading buffer in a 10 mL volumetric flask.

LOADING BUFFER	
Trizma base	0.0788 g
SDS	0.2 g
Bromophenol blue	0.1 g
Milli Q water	7 mL
Glycerol	2 mL

Table 4. Recipe for preparation of separation and loading gel.

	SEPARATION GEL	LOADING GEL
	12.5%	5%
AA/Bis	2.1 mL	0.6 mL
1.88M Tris-HCl	1 mL	
0.625M Tris-HCl		0.8 mL
0.5% SDS	1 mL	0.8 mL
Milli-Q water	0.9 mL	1.8 mL
TEMED	4.15 mL	4 µL
APS	25 µL	10 µL
Total volume	5 mL	2 mL

A silver staining protocol as described in Table 5 was employed in order to visualize the bands of the gel formed from electrophoresis. All the solutions were prepared in 500 mL volumetric flask. Afterwards, the stained gel was scanned (1200 dpi) and transferred to the Quinslab system. The photos were processed and analysed by the ColorTest program that allowed the assignment of densitometric quantifications for each band and the visualization of the bands as electrophoretic graphs.

Table 5. Procedure for silver staining of electrophoresis gel.

	REAGENTS	TIME
Solution 1	5.7 mL acetic acid 32 mL methanol 0.5 mL formaldehyde 37%	45 minutes
Solution 2	250 mL methanol 250 mL milli-Q water	10 minutes, 3 times
Solution 3	0.16 g sodium thiosulfate pentahydrate	1 minute
Rinse	Distilled water	20 seconds
Solution 4	1 g silver nitrate 0.38 mL formaldehyde 37%	20 minutes
Rinse	Distilled water	5 seconds
Solution 5	30 g sodium carbonate 2 mg sodium thiosulfate pentahydrate 0.5 mL formaldehyde 37%	Just until colour change
Rinse	Distilled water	5 seconds
Solution 6	32 mL methanol 5.7 mL acetic acid	1 hour

4 RESULTS AND DISCUSSION

4.1 PREPARATION OF MWCNTS

Over time, very weak sedimentation of MWCNTs in water was observed. This fact proves that carbon nanotubes have been modified on their surface by hydrophilic functional groups. After these modifications, MWCNTs exhibited carboxyl groups on their surface and were prepared for further experimental use and interaction with proteins such as albumin. This technique is known for its reproducibility and simplicity. The oxidized MWCNTs were centrifugated for 3 times to ensure that all acid was removed.

4.2 PROTEIN CORONA ANALYSIS

Protein corona formation is described by an equilibrium process regulated by on-and-off rate coefficients. The first important step of the PC analysis is the incubation of the MWCNTs with BSA before further separation. When nanoparticles are injected in the bloodstream, which contains thousands of proteins, is expected that abundant proteins will associate quickly to the surface of MWCNTs but later they will be substituted by those that bind more tightly (58,94). To evaluate the presence and quantity of protein forming the PC in the samples it was necessary to do several steps: incubate MWCNTs with BSA, separate the unbound proteins from the nanoparticles by centrifugation and washing with PBS, and finally separate and identify the proteins by electrophoresis and photometry. Only tightly binding proteins will continue on the surface of MWCNTs after the separation step (95). Therefore, the proteins that are still adsorbed on the NP after elimination of free proteins and successive washing of the complex represent the hard corona (93). The separation technique that were applied to the samples is influenced by the centrifugation force, the washing solution and duration and the solution volumes on the separation of the freely attached BSA (83).

4.2.1 ELECTROPHORESIS

During the analysis of PC, it is necessary control not only the incubation time but also all factors such as protein source, MWCNTs concentration, temperature and agitation at the thermoblock.

The flow and static conditions must be taken in consideration because the formation of protein corona under dynamic fluidic conditions is clearly different comparing to static conditions. In order to ensure that the *in vivo* conditions are reproduced more accurately, when

the samples were placed in the thermoblock they were under agitation. In capillaries the blood flow velocity ranges from 0.03 cm/sec to 15 cm/sec in both the inferior and superior vena cava. In the aorta the velocity is about 40 cm/sec (79).

Regarding to protein concentration, samples with different concentrations of BSA were prepared. The first sample had not any quantity of protein. In the other samples the amount of albumin were progressively increasing. For each 5 distinct concentrations of BSA (0, 15, 30, 45 and 60 mg/mL), the samples had different incubation times in the thermoblock.

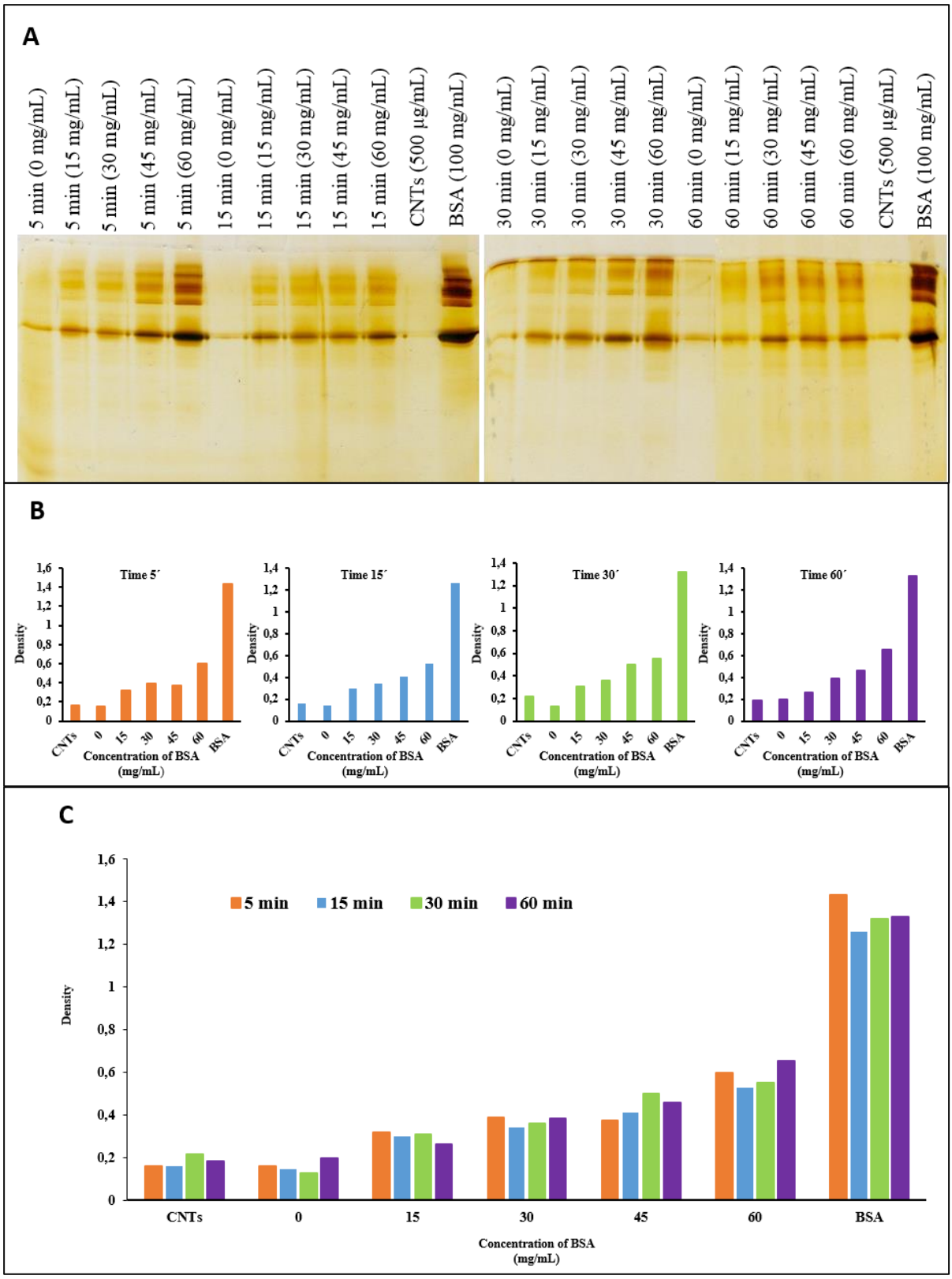

Figure 6. Analysis of the protein corona. Characterization of protein corona formed using the following conditions: MWCNTs (100 µg/mL) and different concentrations of BSA (0, 15, 30, 45, 60 mg/mL) at different times in the thermoblock (5, 15, 30, 60 minutes) at 37 ºC. Negative control was CNTs (500 µg/mL). Positive control was BSA (100 mg/mL). SDS PAGE, 60 min, silver staining (A); densitograms of gels (B) (C).

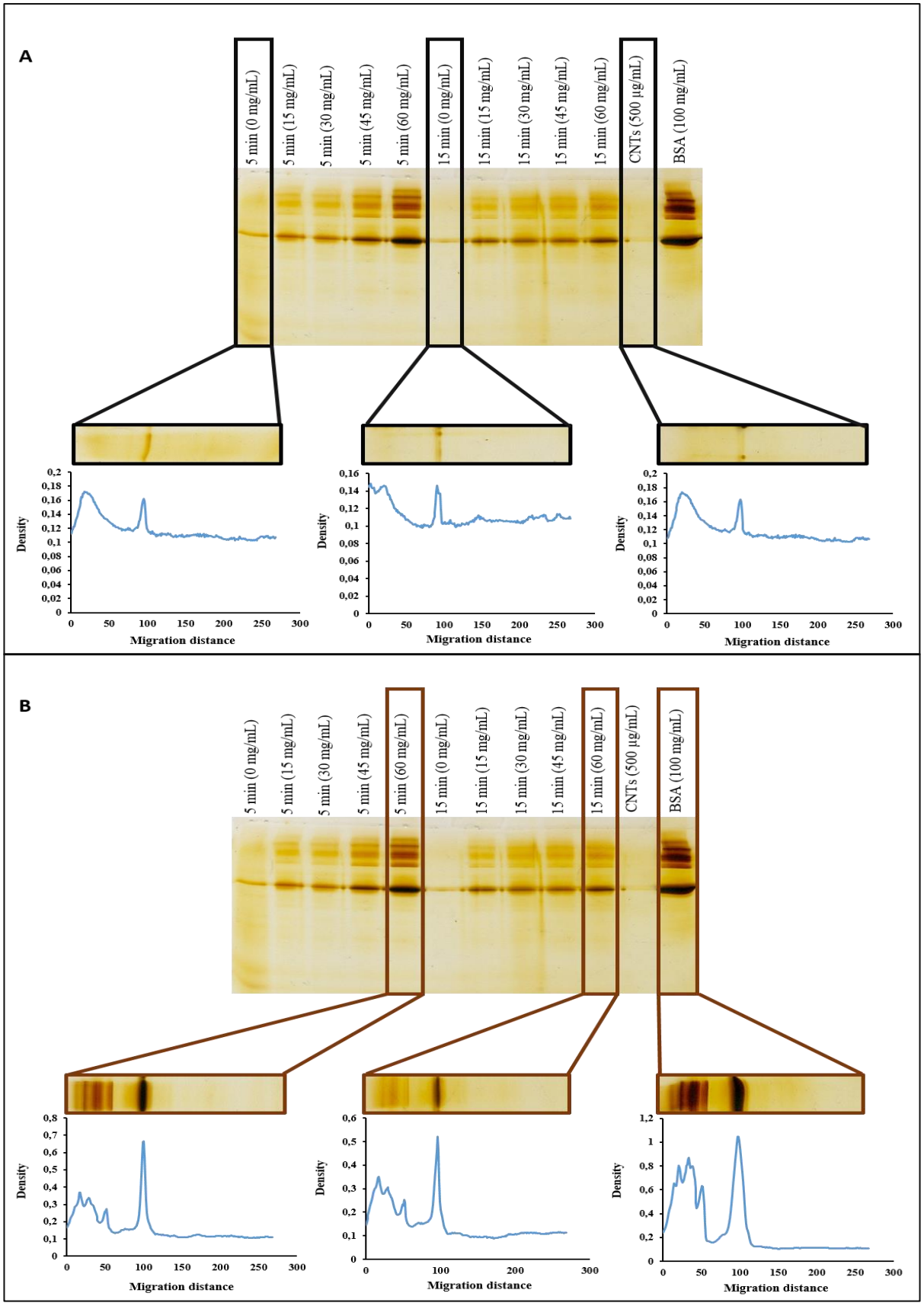

Figure 7. SDS-PAGE of protein corona formed using the following conditions: MWCNTs (100 µg/mL) and different concentrations of BSA (0, 15, 30, 45, 60 mg/mL) at different times in the thermoblock (5, 15, 30, 60 minutes) at 37 ºC. Comparison with the negative control (A); Comparison with the positive control (B).

The formation of protein corona is considerably affected by the protein (BSA) concentration (79). SDS-PAGE gels after silver staining (Figure 7A) proved the formation of MWCNTs-BSA complexes. It is possible to understand that as the concentration of BSA increases, the intensity of the bands become more pronounced. The information given in the gels was converted to graphs by the ColorTest program, which allowed the assignment of densitometric quantifications for each band. Thus, the analysis of the bands is done through the electrophoretic graphs (Figure 7B) that were created by this program. To make the comparison easier, the results obtained were combined in Figure 7C. It is possible to realize in the graph shown in figure 7C that as the BSA concentration increases, the density increases also. Therefore, the amount of BSA is proportional to the formation of protein corona. It is remarkable that the incubation time does not make a significantly difference. When we consider samples that have the same concentration of BSA but different times in the thermoblock, the density not only did not change so much, but also did not always follow the same trend. These results do not agree with what had previously been stated in other experiments. According to other authors, as incubation time increased, the amount of protein corona was expected to increase as well (79,93).

Regardless of the incubation time, when the sample has no BSA, it is not possible to see a clear band and, in opposition, when the sample has the higher concentration of BSA (60 mg/mL) there is a very defined band. To verify the formation and presence of protein corona in our samples, two controls are required: negative (Figure 8A) and positive (Figure 8B). In this case, the negative control is only MWCNTs in PBS, without the presence of albumin. Samples prepared without BSA (BSA 0 mg/mL) at both time 5' and 15' in the thermoblock exhibit an intensity equal to the negative control. The graphs of both samples are also similar to the graph of the negative control, thus confirming the absence of albumin in these prepared samples. In contrast, the positive control is 100 mg/mL of BSA. When comparing the samples with the highest concentration of BSA (60 mg/mL) and the incubation time of 5' and 15', we notice that the intensity of the band is higher than the other samples prepared and in the same place as the positive control. The clear and darker band of the samples with 60 mg/mL of BSA have the same migration distance than positive control, which allows us to conclude that there are proteins in the samples and these proteins are albumin. The presence of BSA in these samples corresponds to protein corona because they are on the surface of MWCNTs. All proteins that did not bind to the carbon nanotubes were eliminated by centrifugation.

The same protocol used to produce the previous samples were also used to prepare other samples with different concentration of carbon nanotubes (50 µg/mL and 200 µg/mL of

oxidized MWCNTs). It was expected to verify a difference in the amount of protein corona when the concentration of carbon nanotubes was changed.

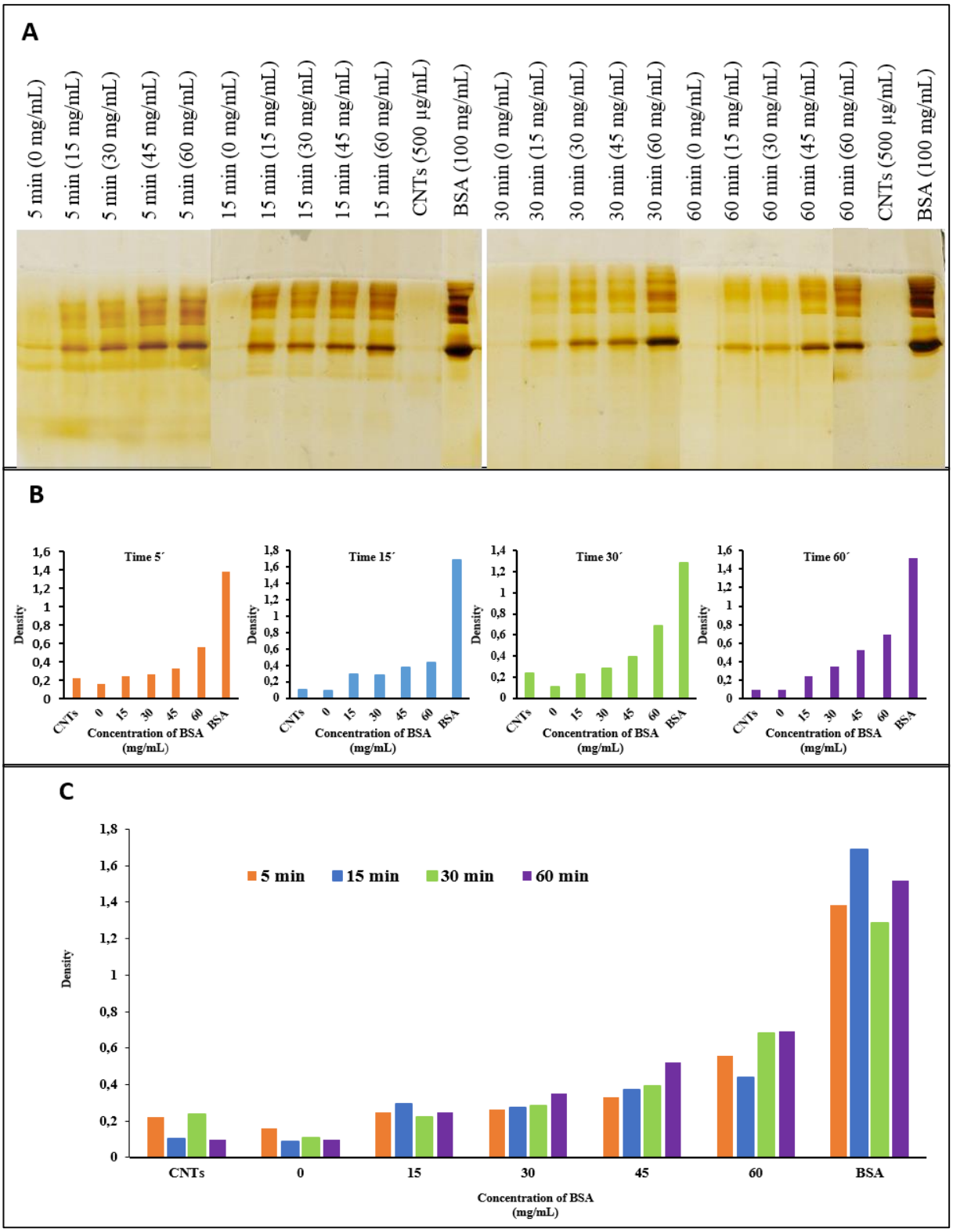

Figure 8. Analysis of the protein corona. Characterization of protein corona formed using the following conditions: MWCNTs (50 µg/mL) and different concentrations of BSA (0, 15, 30, 45, 60 mg/mL) at different times in the thermoblock (5, 15, 30, 60 minutes) at 37 ºC. Negative control was CNTs (500 µg/mL). Positive control was BSA (100 mg/mL). SDS PAGE, 60 min, silver staining (A); densitograms of gels (B) (C).

After the analysis of SDS-PAGE gels (Figure 9A) and the conversion of the information to densitometric graphs (Figure 9B) it is possible to state that the quantity of albumin is proportional to the formation of protein corona as displayed in Figure 9B. Higher concentration of BSA in the samples, more protein corona is formed, as also shown in samples with 100 µg/mL of MWCNTs.

In relation to the time that the samples were exposed to 37ºC, some authors claim that protein corona is instantly formed when CNTs are in contact in blood. Approximately 300 proteins bind to the surface of nanoparticles after only 30 seconds in bloodstream (79,93). The samples with 30 mg/mL and 45 mg/mL of albumin show an increasing tendency for protein corona formation as incubation time increases as demonstrated in Figure 9C. Even if the increase is slight, with these concentrations of albumin is possible to prove that longer incubation time enhances the quantity of protein corona formed.

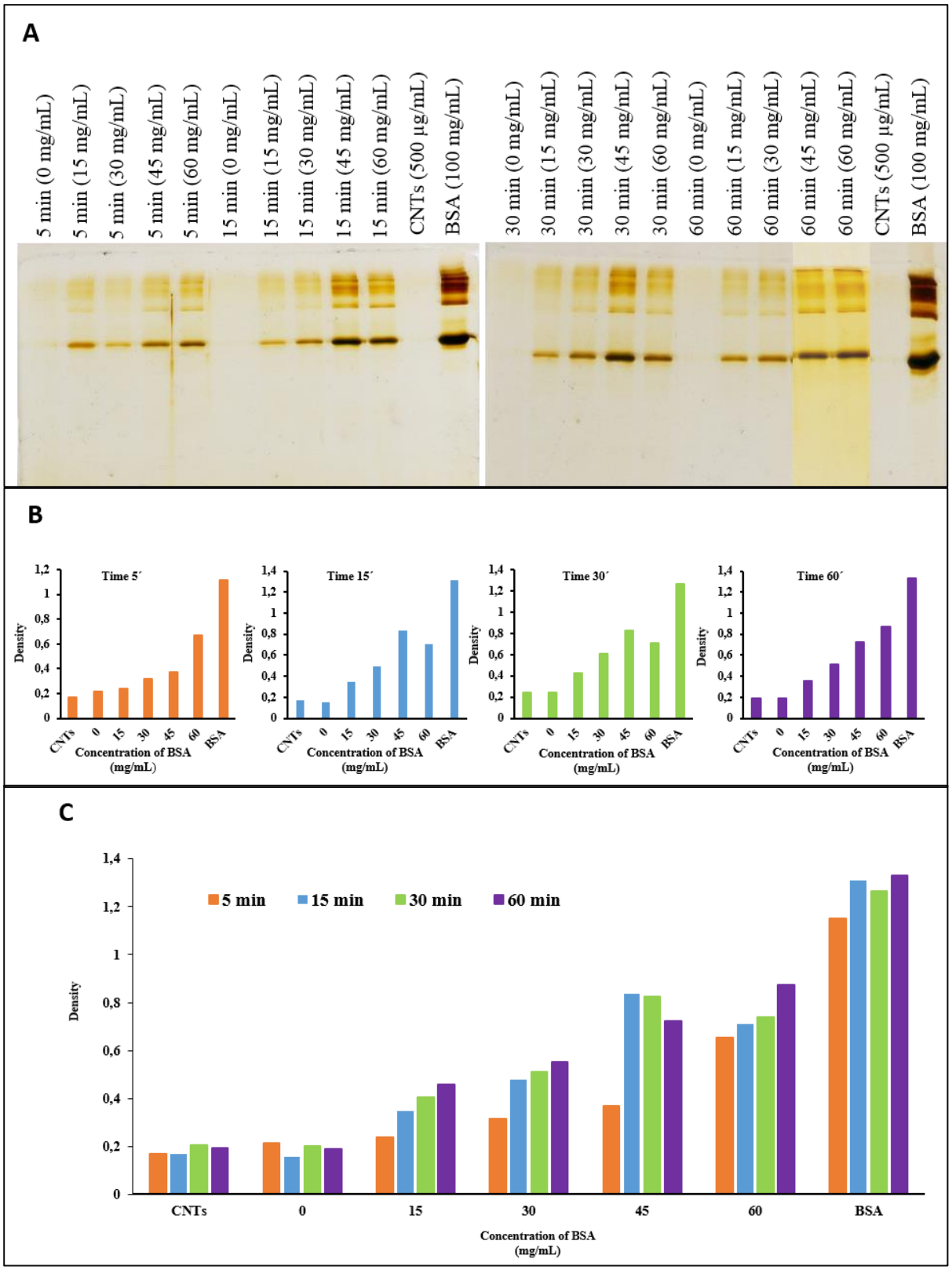

Figure 9. Analysis of protein corona. Characterization of protein corona formed using the following conditions: MWCNTs (200 µg/mL) and different concentrations of BSA (0, 15, 30, 45, 60 mg/mL) at different times in the thermoblock (5, 15, 30, 60 minutes) at 37 ºC. Negative control was CNTs (500 µg/mL). Positive control was BSA (100 mg/mL). SDS PAGE, 60 min, silver staining (A); densitograms of gels (B) (C).

The results obtained by SDS-PAGE (Figure 10A) were converted to densitometric plots (Figure 10B), allowing to evaluate once again the influence of BSA concentration in the formation of protein corona. We can recognize that increasing the concentration of albumin in the samples will increases the density, so more protein corona is formed. In samples with 45 mg/mL of BSA and 15' and 30' at the thermoblock there is a slight increase in density. This can be an experimental error, because in all other samples the trend is the same: more albumin, higher density, so more protein corona is formed. As shown in Figure 10C, incubation time changes the density. With the samples prepared with 15 mg/mL, 30 mg/mL and 60 mg/mL of BSA, it is demonstrated what previous experiments have concluded regarding to increasing incubation time enhances the formation of protein corona (79,93). Although the samples prepared with 45 mg/mL of albumin did not prove this, we can conclude that the incubation time favors the formation of protein corona in this environment conditions (37 ºC and 200 µg/mL).

The concentration of MWCNTs affects the formation of protein corona as demonstrated in Figure 11. According to other authors, there should be an increase in the quantity of protein corona in samples as the amount of MWCNTs enhances.

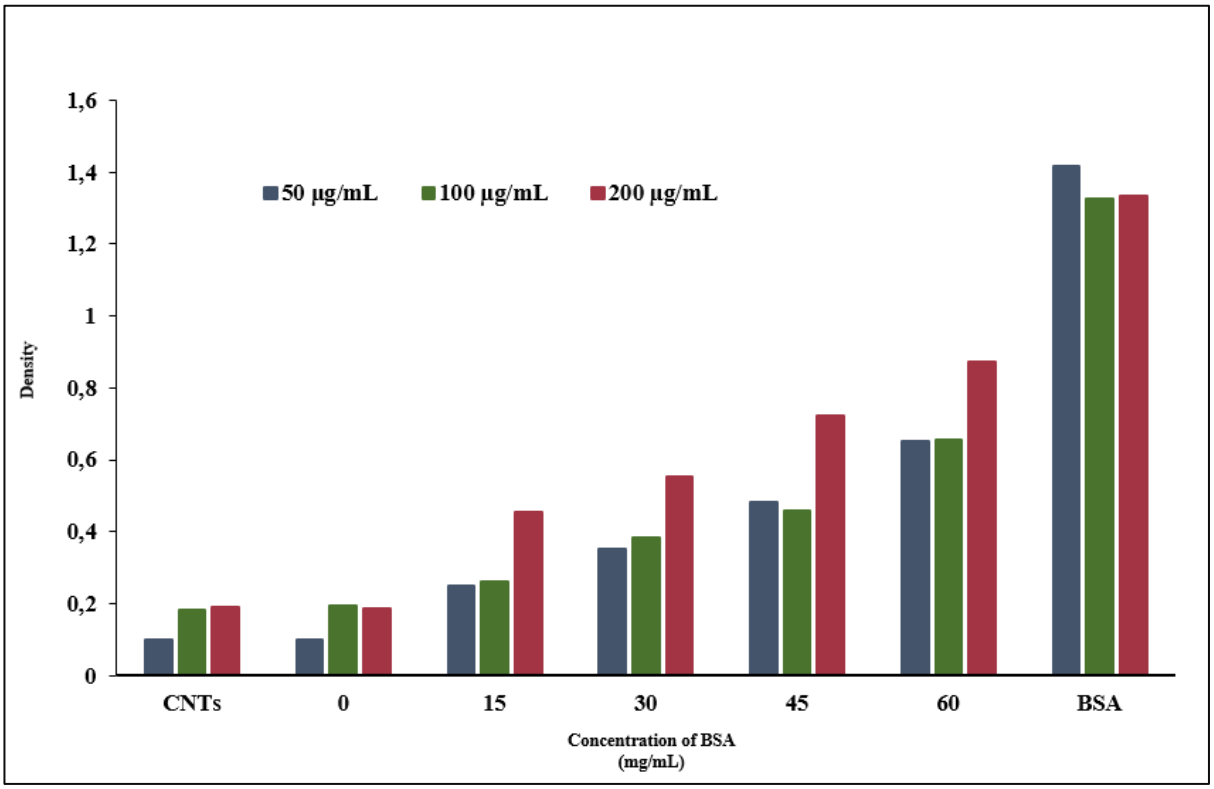

Figure 10. The influence of the concentration of MWCNTs in the formation of protein corona. Summary of the formation MWCNTs-BSA complexes by the interaction between different concentrations of MWCNTs: 50 µg/mL, 100 µg/mL and 200 µg/mL and BSA (0, 15, 30, 45, 60 mg/mL) at 37 ºC and incubation time of 60 minutes.

In this experience, when comparing the different densities presented by samples submitted to 60 minutes in the thermoblock and prepared with various concentrations of carbon nanotubes and albumin we verify that the total of protein corona formed is very similar in the samples with 50 µg/mL and 100 µg/mL of MWCNTs. In opposition, an increase in density is noted when the samples contain 200 µg/mL of MWCNTs. It is possible to conclude that by increasing the concentration of carbon nanotubes from 50 µg/mL to 100 µg/mL there is no difference but increasing to 200 µg/mL there is a significant increase in the quantity of protein corona formed.

Temperature also influences the interaction between carbon nanoparticles and albumin as shown in Figure 12. Several previous studies have shown that the temperature provoked considerable alterations in protein corona pattern after the introduction of MWCNTs in bloodstream. The MWCNTs-BSA complexes altered when exposed to different body temperatures (79). All the results presented above are from samples that were exposed to 37 ºC. In this work we also changed the temperature we subjected the samples in the thermoblock. The protocol used previously was repeated to produce the same samples, but they were submitted to different temperatures in the thermoblock: 30 ºC and 45 ºC.

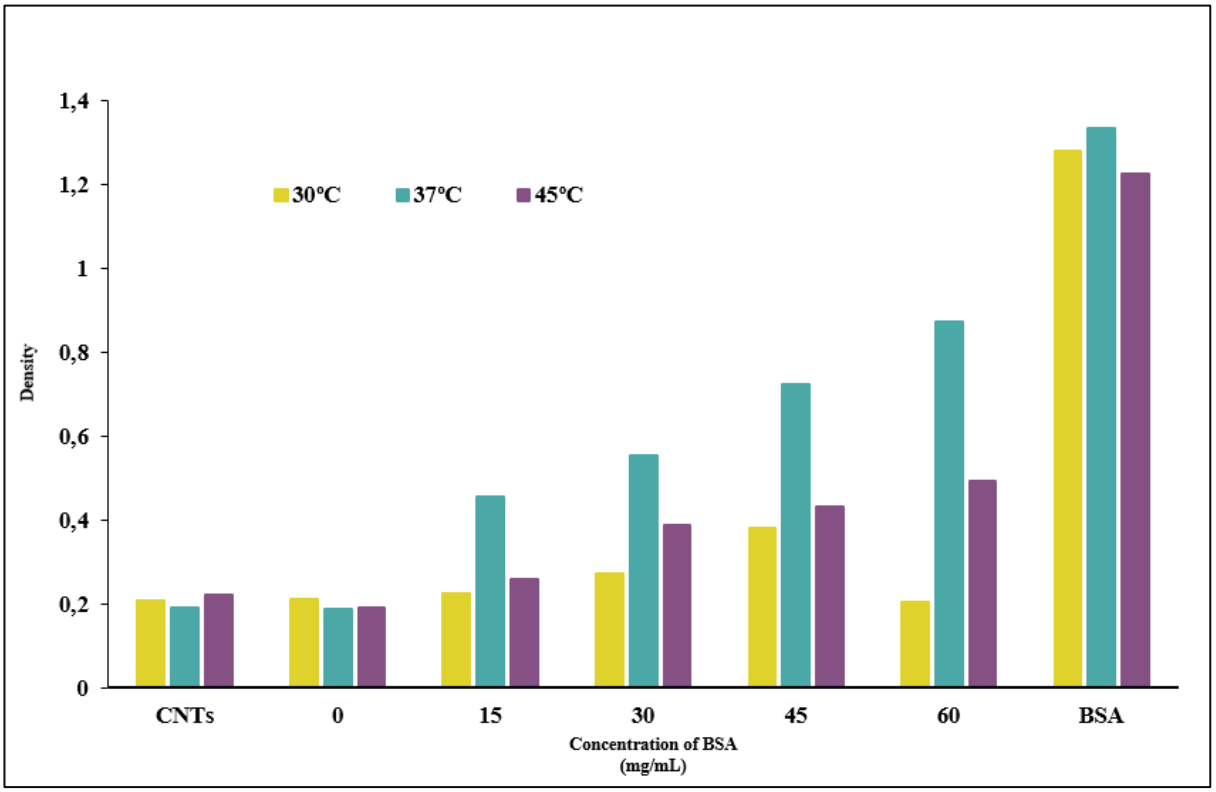

Figure 11. The influence of temperature in the formation of protein corona. Summary of the formation MWCNTs-BSA complexes by the interaction between MWCNTs (200 µg/mL) and BSA (0, 15, 30, 45, 60 mg/mL) at different temperatures: 30 ºC, 37 ºC and 45 ºC and incubation time of 60 minutes.

To prepare the samples we added 3 different concentrations of MWCNTs (50, 100 and 200 µg/mL) to various concentrations of albumin (0, 15, 30, 45, 60 mg/mL) and exposed them for 5, 15, 30 and 60 minutes at two new temperatures: 30 ºC and 45 ºC. By summarizing the information given by the SDS-PAGE gels of these new prepared samples, we can understand that the temperature alters the binding of albumin in the surface of carbon nanotubes. The adsorbed proteins on the nanoparticles surface depend on the temperature but this dependence is not linear (96). The formation of MWCNTs-BSA complexes increases as the temperature rises from 30 ºC to 37 ºC. This increase does not occur when we expose the samples to an even higher temperature: 45 ºC. It is possible to conclude that the greatest formation of protein corona is when the samples are exposed to 37 ºC, so the normal body temperature is the one at which there is the highest adsorption of albumin on the surface of carbon nanotubes. Therefore, the health situation of the patients who will receive the MWCNTs also requires consideration because fever or some diseases can modify the body temperature which changes the interaction between nanoparticles and albumin, leading to alteration in biodistribution and bioavailability (79).

5. CONCLUSION

Anticancer therapy has some problems such as low solubility and bioavailability at tumor tissues. The most of the drug carriers exhibits poor biocompatibility and rapid elimination from the blood circulation (87). As a drug nanocarrier, nanoparticles have several advantages, such as minimize chemoresistance to drug action and reducing their toxicity toward health cells (53). Nanoparticles are also a promising material due to their capacity of targeted delivery of therapeutic drugs to tumor cells in the human body. One approach to achieve the tumorous tissues is to modify and functionalize the surface of the carbon nanotubes with targeting ligands, as albumin, that enhance CNTs binding to receptors on the tumor cells. MWCNTs-albumin conjugates are in the edge over other nanodrugs carriers due to albumin abundance in plasma, albumin stability over a wide range of pH, easy solubility, predictable biodistribution, biocompatibility, capacity to bind to diverse type of drugs, sustained release of drugs and reduced toxicity.

This work concludes that several factors influence the formation of PC. By analysing all samples and considering the different concentrations of MWCNTs and BSA, the incubation times and the temperatures to which samples were subjected, it is realized that in all situations the more albumin, the more PC was formed. The BSA increase is proportional to the formation of PC. Regarding the carbon nanotubes concentration, it also affects the formation of MWCNTs-BSA complexes. The incubation time did not show much influence, contrary to the temperature to which the samples were exposed, being 37 ºC the temperature at which most PC is formed.

A biophysical analysis of albumin was also studied, using the Chemistry Analyser with a photometry method applying either pyrogallol or biuret as reagents. The results were not in agreement with the results from SDS-PAGE due to lack of specificity; it was measured not only the protein concentrations but also the MWCNTs concentration.

In the future, there is no doubt that will exist nanoparticle therapeutics with increasing multifunctionality. With new and more complex nanoparticles conjugates, it will be necessary to create better methodologies to define biocompatibility, especially those that can assess intracellular biocompatibility.

6. FUTURE PERSPECTIVES

Although the use of nanoparticles as drug carrier is already a widely studied and developed subject, there are certain aspects that are not yet clarified. Typically, studies are made on very particular systems and conditions, and the results may not be exchangeable to other systems or even generalizable. It is difficult to simulate all *in vivo* conditions in *in vitro* experiences, because there are several factors to consider. In this work, the next step might be exposing the samples to pH variations by simulating the pH differences that exist between the various organs in human body. The study of pH influence is very important to understand how the release of the anticancer drug from inside the carbon nanotubes occurs. Further studies on solubility and cytotoxicity of carbon nanotubes are needed. For a better study of the formation of MWCNTs-BSA complexes it will be necessary to analyse them using more methods beyond electrophoresis and photometry, such as mass spectrometry techniques.

5 REFERENCES

1. Foulds L. The natural history of cancer. 1958;2–37.
2. Lee BK, Yun YH, Park K, Sturek M. Introduction to biomaterials for cancer therapeutics. Biomater Cancer Ther Diagnosis, Prev Ther. 2013;3–19.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov;394–424.
4. Jung K, Kong H, Won Y-J, Lee ES. Cancer Statistics in Korea : Incidence , Mortality , Survival , and Prevalence in 2016. Korean Cancer Assoc. 2019;51(2):417–30.
5. Stewart BW, Bray F, Forman D, Ohgaki H, Straif K, Ullrich A, et al. Cancer prevention as part of precision medicine : 'plenty to be done.' Oxford Univ Press. 2016;37(1):2–9.
6. Hanahan D, Weinberg RA. The Hallmarks of Cancer. Vol. 100. 2000.
7. Mrsny R. Cancer cell biology. Biomaterials for cancer therapeutics. Woodhead Publishing Limited; 2013. 20–30 p.
8. Marusyk A, Polyak K. Tumor heterogeneity : Causes and consequences. Biochim Biophys Acta - Rev Cancer. 2010;1805:105–17.
9. Desantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, et al. Cancer Treatment and Survivorship Statistics , 2014. CA Cancer J Clin. 2014;252–71.
10. Stylianou A, Lekka M, Stylianopoulos T. AFM assessing of nanomechanical fingerprints for cancer early diagnosis and classification: from single cell to tissue level. R Soc Chem. 2018;20930–45.
11. Wolf F. Methods and systems for treating or preventing cancer. United States Pat. 2016;
12. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global Cancer Statistics , 2012. CA Cancer J Clin. 2015;65(2):87–108.
13. Hecht SS. Tobacco Smoke Carcinogens and Lung Cancer. J Natl Cancer Inst. 1999;91(14):1194–210.
14. Rosato V, Garavello W, Shivappa N, James RH, Serraino D, Vecchia C La. Inflammatory potential of diet and risk of oral and pharyngeal cancer in a large case-

control study from Italy. Int J Cancer. 2017;471–9.

15. Bianchini F, Kaaks R, Vainio H. Overweight, obesity, and cancer risk. Lancet Oncol. 2002;3:565–74.

16. Mao Y, Pan S, Wen SW, Johnson KC. Physical inactivity, energy intake, obesity and the risk of rectal cancer in Canada. Int Union Against Cancer. 2003;831–7.

17. Tomasetti C, Li L, Vogelstein B. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. Am Assoc Adv Sci. 2017;1330–4.

18. Sutton P, Boag JM. Status of vaccine research and development for Helicobacter pylori. Vaccine. 2018;1–5.

19. Schlom J, Gulley JL. Vaccines as an Integral Component of Cancer Immunotherapy. Am Med Assoc. 2018;10–1.

20. Finn OJ. The dawn of vaccines for cancer prevention. Nat Rev Immunol. 2018;18:183–94.

21. Wang T, Wang D, Yu H, Feng B, Zhou F, Zhang H, et al. A cancer vaccine-mediated postoperative immunotherapy for recurrent and metastatic tumors. Nat Commun. 2018 Dec 1;9:1–12.

22. Wang F, Mclafferty S, Escamilla V, Luo L. Late-Stage Breast Cancer Diagnosis and Health Care Access in Illinois. Prof Geogr. 2008;54–69.

23. Kikuyama M, Kamisawa T, Kuruma S, Chiba K, Kawaguchi S, Terada S, et al. Early Diagnosis to Improve the Poor Prognosis of Pancreatic Cancer. 2018;1–9.

24. Yao X, Du N, Hu S, Wang L, Gao J. Rapid advances in research on and development of anticancer drugs in China. Biosci Trends. 2019;1–3.

25. Freeman CR, Farmer J-P. Pediatric Brain Stem Gliomas. J. Radiation Oncology Biol. Phys. 1998.

26. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A Multigene Assay to Predict Recurrence of Tamoxifen-Treated, Node-Negative Breast Cancer. N Engl J Med. 2004;2817–26.

27. Hill MA. Radiation Track Structure: How the Spatial Distribution of Energy Deposition Drives Biological Response. Clin Oncol. 2019 Sep;1–9.

28. Michael BD, Adams GE, Hewitt HB, G Jones WB, Watts ME. A Posteffect of Oxygen in Irradiated Bacteria: A Submillisecond Fast Mixing Study. Vol. 54, Radiation Research. 1973.

29. Nations Scientific Committee on the Effects of Atomic Radiation U. Sources and effects of ionizing radiation. 1993.

30. Chabner BA, Jr TGR. Chemotherapy and the war on cancer. Nat Rev Cancer. 2005;5:65–72.

31. Devita VT, Chu E. A History of Cancer Chemotherapy. Am Assoc Cancer Res. 2008;8643–53.

32. McKnight JA. Principles of Chemotherapy. Clin Tech Small Anim Pract. 2003;18(2):67–72.

33. Papac RJ. Origins of Cancer Therapy. Vol. 74, YALE JOURNAL OF BIOLOGY AND MEDICINE. 2002.

34. Krukiewicz K, Zak JK. Biomaterial-based regional chemotherapy: Local anticancer drug delivery to enhance chemotherapy and minimize its side-effects. Mater Sci Eng C. 2016;62:927–42.

35. Menchón SA. The Effect of Intrinsic and Acquired Resistances on Chemotherapy Effectiveness. Acta Biotheor. 2015;63:113–27.

36. Vanneman M, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. Vol. 12, Nature Reviews Cancer. 2012. p. 237–51.

37. Corrie PG. Cytotoxic chemotherapy: Clinical aspects. 2016;25–8.

38. Chen Y, Ph D, Chang M, Ph D, Cheng W, Ph D. Metronomic Chemotherapy and Immunotherapy in Cancer Treatment. Cancer Lett. 2017;1–51.

39. Blattman JN, Greenberg PD. Cancer Immunotherapy : A Treatment for the Masses. Am Assoc Adv Sci. 2004;305:200–5.

40. Hughes B. Antibody-drug conjugates for cancer: poised to deliver? Vol. 9, Nature Reviews Drug Discovery. Nature Publishing Group; 2010. p. 665–7.

41. Diamantis N, Banerji U. Antibody-drug conjugates - An emerging class of cancer treatment. Vol. 114, British Journal of Cancer. Nature Publishing Group; 2016. p. 362–7.

42. Meier DE, Brawley OW. Palliative Care and the Quality of Life. J Clin Oncol. 2011;29:2750–2.

43. Siegel R, Desantis C, Virgo K, Stein K, Mariotto A, Smith T, et al. Cancer Treatment and Survivorship Statistics , 2012. CA Cancer J Clin. 2012;62:220–41.

44. Foley KM. The treatment of cancer pain. N Engl J Med. 1985;

45. Barbera L, Seow H, Howell D, Sutradhar R, Earle C, Liu Y, et al. Symptom burden and performance status in a population-based cohort of ambulatory cancer patients. 2010 Dec 15;5767–76.

46. Oun R, Moussa YE, Wheate NJ. The side effects of platinum-based chemotherapy drugs: A review for chemists. R Soc Chem. 2018;47(19):6645–53.

47. Brannon-Peppas L, Blanchette JO. Nanoparticle and targeted systems for cancer therapy. Adv Drug Deliv Rev. 2004;1649–59.

48. Marciel kelli K. Symptom management in cancer: Pain, depression, and fatigue. J Natl Cancer Inst. 2003 Aug 6;95(15):1110–7.

49. Lorusso D, Bria E, Costantini A, Di Maio M, Rosti G, Mancuso A. Patients' perception of chemotherapy side effects: Expectations, doctor–patient communication and impact on quality of life – An Italian survey. Eur J Cancer Care (Engl). 2016;1–9.

50. Aigner KR, Stephens FO. Induction Chemotherapy. 2011. 1–360 p.

51. Ferrari M. Cancer nanotechnology: opportunities and challenges. Nat Rev Cancer. 2005;5.

52. Farokhzad OC, Langer R. Impact of Nanotechnology on Drug Delivery. Am Chem Soc. 2009;3:16–20.

53. Kumari P, Ghosh B, Biswas S. Nanocarriers for cancer-targeted drug delivery. J Drug Target. 2015;1–13.

54. Capjak I, Goreta SŠ, Jurašin DD, Vrček IV. How protein coronas determine the fate of engineered nanoparticles in biological environment. 2017;245–53.

55. Lane LA, Qian X, Smith AM, Nie S. Physical Chemistry of Nanomedicine : Understanding the Complex Behaviors of Nanoparticles in Vivo. Annu Rev ofPhysical Chem. 2015;521–47.

56. Jurašin DD, Ćurlin M, Capjak I, Crnkovi T, Lovri M, Babi M, et al. Surface coating affects behavior of metallic nanoparticles in a biological environment. Beilstein J Nanotechnol. 2016;246–62.

57. Durán N, Silveira CP, Durán M, Martinez DST. Silver nanoparticle protein corona and toxicity : a mini - review. J Nanobiotechnology. 2015;1–17.

58. Docter D, Westmeier D, Markiewicz M, Stolte S, Knauer SK. The nanoparticle biomolecule corona : lessons learned – challenge accepted? R Soc Chem. 2015;44:6094–121.

59. Davis ME, Chen ZG, Shin DM. Nanoparticle therapeutics : an emerging treatment modality for cancer. Nat Rev Drug Discov. 2008;7:771–82.

60. Mirshafiee V, Mahmoudi M, Lou K, Cheng J, Kraft ML. Protein Corona Significantly Reduces Active Targeting Yield. Natl Institutes Heal. 2013;1–9.

61. Biswas S, Torchilin VP. Nanopreparations for Organelle-Specific Delivery in Cancer. NIH Public Access. 2015;1–36.

62. Li Y, Wang J, Wientjes MG, Au JL. Delivery of nanomedicines to extracellular and intracellular compartments of a solid tumor. Adv Drug Deliv Rev. 2012;64:29–39.

63. Ozcelikkale A, Ghosh S, Han B. Multifaceted Transport Characteristics of Nanomedicine: Needs for Characterization in Dynamic Environment. Am Chem Soc. 2013;2111–26.

64. Rizzo LY, Theek B, Storm G, Kiessling F, Lammers T. Recent progress in nanomedicine : therapeutic , diagnostic and theranostic applications. Curr Opin Biotechnol. 2013;24:1159–66.

65. Hosnedlova B, Kepinska M, Fernandez C, Peng Q, Ruttkay-Nedecky B, Milnerowicz H, et al. Carbon Nanomaterials for Targeted Cancer Therapy Drugs : A Critical Review. Chem Rec. 2019;19:502–22.

66. Torchilin V. Tumor delivery of macromolecular drugs based on the EPR effect. Adv

Drug Deliv Rev. 2011;63:131–5.

67. Rolfo C, Giovannetti E. A synthetic lethal bullet. Nat Nanotechnol. 2017;

68. Serpe L. Conventional Chemotherapeutic Drug Nanoparticles for Cancer Treatment. Vol. 6. 2006. 1–39 p.

69. Maeda H, Sawa T, Konno T. Mechanism of tumor-targeted delivery of macromolecular drugs , including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. J Control Release. 2001;74:47–61.

70. Torchilin VP. Passive and Active Drug Targeting: Drug Delivery to Tumors as an Example. 2010. 3–53 p.

71. Maeda H, Bharate GY, Daruwalla J. Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. Eur J Pharm Biopharm. 2009;71:409–19.

72. Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev. 2002;631–51.

73. Cho K, Wang X, Nie S, Chen Z, Shin DM. Therapeutic nanoparticles for drug delivery in cancer. Clin Cancer Res. 2008;14:1310–6.

74. L. Arias J. Drug Targeting Strategies in Cancer Treatment: An Overview. Mini-Reviews Med Chem. 2010;11:1–17.

75. Dlamini N, Mukaya HE, Van Zyl RL, Chen CT, Zeevaart RJ, Mbianda XY. Synthesis, characterization, kinetic drug release and anticancer activity of bisphosphonates multi-walled carbon nanotube conjugates. Mater Sci Eng C. 2019;1–9.

76. Cai X, Ramalingam R, Wong HS, Cheng J, Ajuh P, Cheng SH, et al. Characterization of carbon nanotube protein corona by using quantitative proteomics. Nanomedicine Nanotechnology, Biol Med. 2013;9:583–93.

77. Zhang T, Tang M, Yao Y, Ma Y, Pu Y. MWCNT interactions with protein : surface-induced changes in protein adsorption and the impact of protein corona on cellular uptake and cytotoxicity. Int J Nanomedicine. 2019;14:993–1009.

78. Zanganeh S, Spitler R, Erfanzadeh M, Alkilany AM, Mahmoudi M. Protein corona: Opportunities and challenges. Int J Biochem Cell Biol. 2016;75:143–7.

79. Nguyen VH, Lee BJ. Protein corona: A new approach for nanomedicine design. Int J

Nanomedicine. 2017;12:3137–51.

80. Mariam J, Sivakami S, Dongre PM. Albumin corona on nanoparticles–a strategic approach in drug delivery. Drug Deliv. 2016;23:2668–76.

81. Corbo C, Molinaro R, Parodi A, Toledano Furman NE, Salvatore F, Tasciotti E. The impact of nanoparticle protein corona on cytotoxicity, immunotoxicity and target drug delivery. Nanomedicine. 2016;11:81–100.

82. Charbgoo F, Nejabat M, Abnous K, Soltani F, Mohammad S, Alibolandi M, et al. Gold nanoparticle should understand protein corona for being a clinical nanomaterial. J Control Release. 2018;272:39–53.

83. Zanganeh S, Ho JQ, Aieneravaie M, Erfanzadeh M, Spitler R. Protein Corona: The Challenge at the Nanobiointerfaces. Iron Oxide Nanoparticles Biomed Appl. 2018;91–104.

84. Peng Q, Zhang S, Yang Q, Zhang T, Wei XQ, Jiang L, et al. Preformed albumin corona, a protective coating for nanoparticles based drug delivery system. Biomaterials. 2013;34:8521–30.

85. Singh P, Singh H, Ahn S, Kim YJ, Yang DC. Bovine serum albumin as a nanocarrier for the e ffi cient delivery of ginsenoside compound K : preparation , physicochemical characterizations and in vitro biological studies. R Soc Chem. 2017;7:15397–407.

86. Merlot AM, Kalinowski DS, Richardson DR. Unraveling the mysteries of serum albumin-more than just a serum protein. Front Physiol. 2014;5:1–7.

87. Zhao P, Wang Y, Wu A, Rao Y, Huang Y. Roles of Albumin-Binding Proteins in Cancer Progression and Biomimetic Targeted Drug Delivery. ChemBioChem. 2018;19:1796–805.

88. Hoogenboezem EN, Duvall CL. Harnessing albumin as a carrier for cancer therapies. Adv Drug Deliv Rev. 2018;130:73–89.

89. Fatkhutdinova LM, Khaliullin TO, Vasil'yeva OL, Zalyalov RR, Mustafin IG, Kisin ER, et al. Fibrosis biomarkers in workers exposed to MWCNTs. Toxicol Appl Pharmacol. 2016;1–22.

90. Carrillo-carrion C, Carril M, Wolfgang J. Techniques for the experimental investigation

of the protein corona. Curr Opin Biotechnol. 2017;46:106–13.

91. Hackney AC. Athlete Testing, Analytical Procedures, and Adverse Analytical Findings. Doping, Performance Enhancing Drugs, and Hormones in Sport. 2018. 113–127 p.

92. Stewart D, Dhungana S, Clark R, Pathmasiri W, McRitchie S, Sumner S. Omics Technologies Used in Systems Biology. Systems Biology in Toxicology and Environmental Health. Elsevier Inc.; 2015. 57–83 p.

93. Weber C, Morsbach S, Landfester K. Possibilities and Limitations of Different Separation Techniques for the Analysis of the Protein Corona. Angew Chemie Int Ed. 2019;58:12787–94.

94. Monopoli MP, Åberg C, Salvati A, Dawson KA. Biomolecular coronas provide the biological identity of nanosized materials. Nat Nanotechnol. 2012;7:779–86.

95. Wang H, Lin Y, Nienhaus K, Nienhaus GU. The protein corona on nanoparticles as viewed from a nanoparticle-sizing perspective. WIREs Nanomedicine and Nanobiotechnology. 2018;10:1–9.

96. Mahmoudi M, Abdelmonem AM, Behzadi S, Clement JH, Dutz S, Ejtehadi MR, et al. Temperature - The "Ignored" Factor at the NanoBio Interface. Am Chem Soc. 2013;1–20.